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# Processing and glycosylation of CD46 regulate its expression and T cell responses

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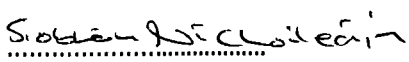
# Declaration

I declare that this thesis is my own work, apart from the help acknowledged herein. The work within this thesis has not been submitted for any other degree or professional qualification, except as specified.



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# Abbreviations

| | |
|---|---|
| Ad35 | adenovirus type 35 |
| ADAM | a Disintegrin and metalloproteinase domain-containing protein |
| aHUS | atypical haemolytic uremic syndrome |
| APC | allophycocyanin |
| APC | antigen presenting cell |
| APP | amyloid precursor protein |
| BBB | blood brain barrier |
| BSA | bovine serum albumin |
| CFSE | carboxyfluorescein diacetate succinimidyl ester |
| CHO | Chinese hamster ovary |
| CNS | central nervous system |
| CTF | cytoplasmic transmembrane fragment |
| CTF1 | cytoplasmic transmembrane fragment-Cyt1 |
| CTF2 | cytoplasmic transmembrane fragment-Cyt2 |
| CTL | cytotoxic lymphocyte |
| CTLA4 | cytotoxic T-lymphocyte antigen 4 |
| CVO | control vector only |
| DAPT | N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycinet-Butyl Ester |
| EAE | experimental autoimmune encephalomyelitis |
| EBV | Epstein–Barr virus |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| FITC | fluorescein isothiocyanate |
| FoxP3 | factor forkhead box P3 |
| GVHD | graft-versus-host disease |
| GWAS | genome wide association studies |

| | |
|---|---|
| HHV-6 | human herpes virus 6 |
| HRP | horseradish peroxidase |
| HUS | hemolytic uremic syndrome |
| ICD | intracellular domain |
| IFN | interferon |
| IP | immunoprecipitation |
| iTreg | inducible T regulatory cell |
| MBP | myelin basic protein |
| MHC | major histocompatability complex |
| MMP | metalloproteinase |
| MOG | myelin oligodendrocyte glycoprotein |
| MS | multiple sclerosis |
| MV | measles virus |
| MVB | multivesicular bodies |
| $M_w$ | molecular weight |
| NMFI | normalised mean fluorescence intensity |
| nTreg | natural T regulatory cell |
| p-LAT | phosphorylated linker activation |
| PAMPS | patterns associated with microbial pathogens |
| PBMC | peripheral blood mononuclear cells |
| PE | phycoerythrin |
| PGE-2 | prostaglandin E2 |
| PRR | pathogen recognition receptors |
| PγS | presenilin-γ-secretase |
| RA | rheumatoid arthritis |
| RIPA | radio immunoprecipitation assay buffer |
| RRMS | relapsing-remitting multiple sclerosis |
| sCD46 | soluble CD46 |

| | |
|---|---|
| SCID | severe combined immunodeficiency |
| SCR | short consensus repeat |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SLE | systemic lupus erythematosus |
| STAT | signal transducer and activators of transcription |
| STP | serine, theronine and proline |
| T-bet | T-box expressed in T cells |
| TCL | total cell lysate |
| TCR | T cell receptor |
| Th | helper T cell |
| TMB | tetramethylbenzidine |
| Tr1 | type I regulatory T cells |
| Tregs | T regulatory cells |
| UNCL.F1 | uncleavable cytoplasmic transmembrane fragment-Cyt1 |
| UNCL.F2 | uncleavable cytoplasmic transmembrane fragment-Cyt2 |
| US | unstimulated |

# Abstract

CD46 is a ubiquitously expressed transmembrane molecule and has an important role in the innate and adaptive immune system. CD46 was originally identified as a complement receptor that protects cells from autologous attack. However, CD46's immunological profile is ever expanding and more recently it was identified as a T cell costimulatory molecule. Notably, in the presence of IL-2, CD46 can induce a Tr1-like phenotype that is characterized by the secretion of large amounts of the potent anti-inflammatory cytokine, IL-10. Defects in CD46-induced IL-10 secretion have been identified in multiple sclerosis, asthma and rheumatoid arthritis. Despite CD46's promiscuous nature in immune responses there is little known about its underlying processing and signalling pathways.

Herein, I report that CD46 expression and processing are important for regulating T cell anti-inflammatory responses and activation. Cyt1 but not Cyt2 promotes an increased ratio IL-$10^{+}$ cells compared to cells that secrete both Il-10 and IFNγ. Upon CD46 costimulation, proteolytic cleavage of CD46 occurs at the surface and intracellularly with the subsequent release of a functional intracellular domain (ICD). As a result of alternative splicing, there are two main cytoplasmic isoforms of CD46, both of which release ICDs, Cyt1 and Cyt2. It is shown that the smaller Cyt1 ICD fragment facilitates T cell activation, whereas, Cyt2 promotes T cell activation when expressed in an uncleaved form.

As the expression and cleavage of CD46 is important for regulating T cell function, I went on to identify factors that can regulate CD46 cleavage. Herein, it is demonstrated that T cell activation by the T cell receptor (TCR) acts as a major regulator of CD46 cleavage and expression, emphasizing the inherent role of CD46 in T cell activation. TCR stimulation also modulates CD46 glycosylation, which may effect CD46 expression and T cell phenotype. Importantly, I have identified a dysregulated expression of CD46 in a preliminary cohort of RRMS

patients. It will be interesting to examine if aberrant CD46 glycosylation or cleavage accounts for its altered expression levels and impaired IL-10 secretion in RRMS patients.

# Chapter 1 : Introduction

*CD46 is a 'pathogen magnet' and not without reason: understanding how CD46 stands on the cross roads of innate and adaptive immunology will open the door to a more integrated view of the immune system during health and disease.*

## 1.1 The inflammatory response

A healthy immune system regulates pathogen infection while maintaining tolerance to self (Sakaguchi et al., 2008, Bluestone, 2011). In vertebrates, the immune system consists of two elements, the innate and the adaptive immune response. The innate system is responsible for the initial detection of pathogens through the expression of germ-line encoded pathogen recognition receptors (PRR), which detect conserved patterns associated with microbial pathogens (PAMPS) (Medzhitov and Janeway, 1998, Janeway and Medzhitov, 2002). PAMPS are only expressed on microorganisms and therefore host antigens do not activate PRRs. Innate immune cells include macrophages, dendritic cells and neutrophils. Once innate immune cells have encountered PAMPS they become activated and their effector mechanisms are initiated. These mechanisms include phagocytosis of pathogens, cytokine secretion and the activation of the adaptive immune response (Medzhitov and Janeway, 1998, Abbas and Janeway, 2000, Janeway and Medzhitov, 2002). The adaptive immune response is acquired through one's lifetime and retains memory of previous pathogens encountered (Abbas and Janeway, 2000, Sprent and Surh, 2002). The cells of the adaptive immune response are the T and B cells. T cells are activated by antigen presenting cells (APCs), which include the dendritic cells and macrophages of the innate immune system (Medzhitov and Janeway, 1998, Abbas and Janeway, 2000). In order to recognise pathogens, T cells utilize receptors (TCRs) that are created by a somatic process of random gene rearrangement to recognise pathogenic antigens. Each TCR generated by gene rearrangement is believed to be cross-reactive with up to 1 million peptides. As a result, TCRs are capable of

detecting a massive range of potential pathogens (Mason, 1998, Sewell, 2012). There are two subgroups of T cells: the $CD8^+$ cytotoxic lymphocytes (CTLs) and the $CD4^+$ helper T cells ($T_h$). CTLs directly target infected cells that are presenting their cognate antigen and induce cell death, whereas the $T_h$ cells as the name suggests, help to guide other leukocytes during the immune response. $T_h$ cells have two main functions, to induce B cell activation and to secrete cytokines, which is fundamental to the regulation of the immune response. Once B cells are activated they produce high quantities of antibodies specific to the pathogen. These antibodies spread throughout the body, inhibiting pathogen function or labelling them for phagocytosis by the innate cells. Therefore, the innate and adaptive immune systems work together to provide a highly efficient defence against pathogens (Medzhitov and Janeway, 1998, Abbas and Janeway, 2000). Indeed, the immune system is a complex system and important regulatory mechanisms must be in place to control its function.

When the immune system is not correctly regulated it can give rise to both autoimmunity and allergy. To prevent this from happening there are a series of checkpoints in lymphocyte development – these occur in the thymus (central tolerance) and in the periphery (peripheral tolerance) (Bluestone, 2011). Within the thymus, the population of T cells is enriched with T cells that are only reactive to antigen that is presented in the context of the host's major histocompatability complex (MHC) receptors. MHC receptors help the host's immune system differentiate between self and pathogen. T cells that cannot bind to the host's MHC cannot interact with APCs and are therefore eliminated in a process known as positive selection (Medzhitov and Janeway, 1998, Abbas and Janeway, 2000). The T cells that are positively selected are reactive to both pathogens and self. In order to prevent the self-reactive T cells from being activated in the periphery and causing unwanted inflammatory reactions, the majority of these highly self-reactive cells undergo cell death in a process called negative selection (Medzhitov and Janeway, 1998, Abbas and Janeway, 2000, Mueller, 2010, Bluestone, 2011). However, this process does not eliminate all

self-reactive T cells and these cells escape into the periphery as mature T cells. Within the periphery there are further processes in place, which not only prevent the activation of self-reactive T cells but also control inflammatory responses to pathogens (Mueller, 2010, Goverman, 2011, Bluestone, 2011).

The first basic mechanism of control in the periphery is the requirement of the T cell to receive two activating signals from the APC. First, for a T cell to become activated it needs to encounter its cognate antigen in context of the appropriate MHC this is called "signal 1". However, a T cell must also receive a costimulatory signal from the APC called "signal 2" (*figure 1.1*) (Janeway, 1989). The most well studied costimulatory pathway involves the T cell costimulatory receptor CD28 and its ligands CD80 (B7-1) and CD86 (B7-2) that are expressed on professional APCs (Sperling and Bluestone, 1996). The costimulatory stimulus is required for full activation of a T cell and if signal 2 is not received the T cell becomes functionally inactive or anergic (Sperling and Bluestone, 1996, Medzhitov and Janeway, 1998, Abbas and Janeway, 2000, Mueller, 2010). Of note, other costimulatory receptors are also important for T cell activation including; ICOS, OX40, 4-1BB and CD2 (Leitner et al., 2010). The focus of this project is on the more recently discovered T cell costimulatory receptor CD46 (Astier et al., 2000) (*figure 1.1*)

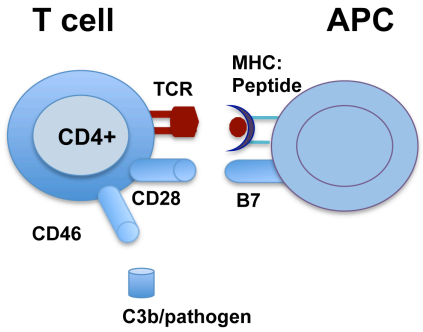


***Figure 1.1: The two-signal model of T cell activation.*** *Signal 1 involves the interaction between the TCR and its cognate peptide:MHC complexes. Signal 2 is received by a costimulatory stimulus; the costimulatory molecules CD28 and CD46 are shown with their respective ligands.*

Once T cells are activated they act to co-ordinate an inflammatory response against invading pathogens. In order to prevent the activation of self-reactive T cells or excessive immune responses, a second important mechanism of peripheral tolerance is required: T regulatory cells (Tregs) (Sakaguchi et al., 1995). Tregs are a subset of T cells that can prevent excessive immune responses to pathogens or the activation of self-reactive T cells. Specifically, Tregs suppress the effector function of several immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, NKT cells, B cells, macrophages and dendritic cells (Sakaguchi et al., 2008, Sakaguchi et al., 2010). The function of Tregs is known to be dysfunctional in several inflammatory diseases, including multiple sclerosis (MS) and the mechanisms of their functions are under intensive investigation (Sakaguchi, 2004, Wraith et al., 2004, Roncarolo et al., 2006, Costantino et al., 2008b, Sakaguchi et al., 2010).

## 1.2 CD4+ T cell Subsets

$CD4^+$ T cells are a fundamental part of the adaptive immune response as they can orchestrate the role of other adaptive and innate immune cells. There are several subsets of $CD4^+$ T cells, which have been characterised based on their specific function *in vivo* (Mosmann et al., 1986, Sakaguchi et al., 1995, Groux et al., 1997, Harrington et al., 2006). Generally, prior to encountering its cognate antigen a T cell is referred to as a naïve T cell and does not yet have a specific role. However, upon antigen recognition, a naïve T cell receives a range of cues from the antigen interaction itself, cytokines, and costimulatory and adhesion molecules. The combination of these signals results in the activation of sets of transcription factors that regulate gene expression to shape T cell differentiation and function. *Mossman et al* first described the first two recognised subsets: Th1 and Th2 (Mosmann et al., 1986). However, the number of subsets later expanded to include Tregs (Sakaguchi, 2004, Groux et al., 1997) and Th17 cells (Langrish et al., 2005, Harrington et al., 2006) (*figure 1.2*). Both Th1 and Th17 cells are

thought to play an important role in the pathogenesis of MS, while Tregs play a protective role (Costantino et al., 2008a, Petermann and Korn, 2011, Nylander and Hafler, 2012). These T cell subsets are described below.

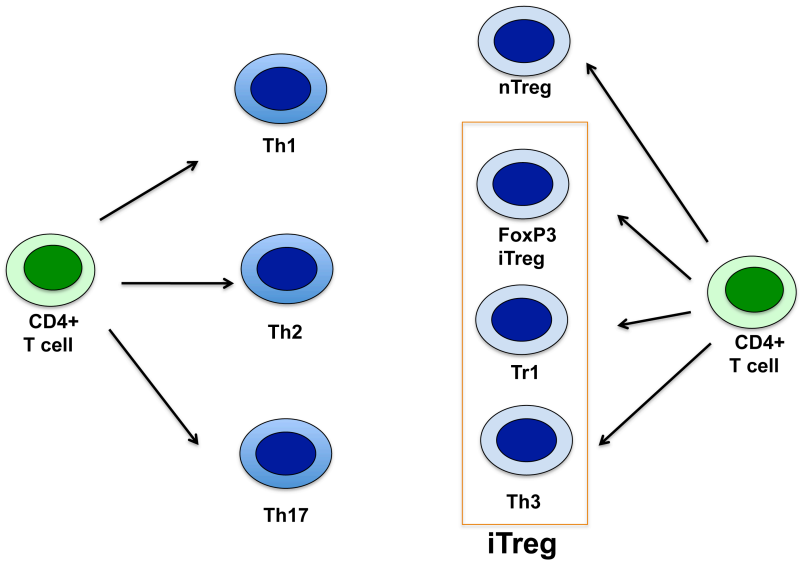


***Figure 1.2: A schematic representing the relevant and well-described CD4$^+$ T cell subsets.***

### 1.2.1 Th1

The Th1 T cell subset is characterised by their secretion of interferon-γ (IFNγ) and their specific function of targeting intracellular pathogens such as *Mycobacterium tuberculosis* (Mosmann et al., 1986, Mosmann and Coffman, 1989). Naïve T cells are driven into the Th1 lineage in the presence of IL-12 and IFNγ. These cytokines result in the activation of signal transducer and activators of transcription (STAT) 1 and STAT4 leading to the upregulation of the transcription factor, T-box expressed in T cells (T-bet) (Hsieh et al., 1993, Nishikomori et al., 2002, Hibbert et al., 2003). T-bet is described as the Th1 'master regulator', a key marker for Th1 differentiation. T-bet inhibits both Th2 and Th17 responses, and promotes IFNγ production providing a positive feedback loop for Th1 commitment (Kanno et al., 2012). In addition to autoregulation, IFNγ also activates macrophages, cytotoxic T cells and natural

killer T cells and antibody IgG2a production by B cells (Petermann and Korn, 2011). This subset also produces IL-2, TNF-α, GM-CSF, lymphotoxin, IL-10 but no IL-4, the trademark cytokine of Th2 differentiated cells (Mosmann et al., 1986, O'Garra and Vieira, 2007). Of note, dysregulated Th1 responses have traditionally been associated with chronic inflammation and tissue destruction (Petermann and Korn, 2011).

### 1.2.2 Th17

The Th17 T cell subset is a more recently identified lineage of effector T cells (Langrish et al., 2005, Harrington et al., 2006). This subset is characterised by IL-17 production and is crucial to the control of extracellular fungi, bacterial infections and microbes that are not targeted by Th1 or Th2 effector T cells (Harrington et al., 2006).The Th17 phenotype is promoted in the presence of TGFβ, IL-1β, IL-6 and IL-21 or IL-23 (Manel et al., 2008). The 'master regulator' of Th17 differentiation is the transcription factor, RORγt, which works in concert with RORα to drive Th17 lineage commitment and IL-17 secretion (Yang et al., 2008). IL-17 can induce the release of inflammatory cytokines and chemokines from a wide variety of cells and plays an important role in the recruitment of neutrophils (Petermann and Korn, 2011). Of note, both Th1 and Th2 trademark cytokines, IFNγ and IL-4 strongly suppress Th17 differentiation (Harrington et al., 2006). However Th17 subsets are not as stable as Th1 and Th2 subsets and this has raised questions about their existence as a distinct lineage (Petermann and Korn, 2011). Nonetheless, due to the pro-inflammatory nature of Th17 cytokines, they have become an important target in the understanding and treatment of autoimmune diseases. For example, Th17 related cytokines, IL-23 and IL-17, have been shown to play a pivotal role in some experimental autoimmune encephalomyelitis (EAE) models and collagen-induced arthritis (Harrington et al., 2006, Petermann and Korn, 2011, Nylander and Hafler, 2012).

## 1.2.3 Treg

As previously mentioned, whilst inflammatory responses are key to keeping pathogens under control, there are also mechanisms in place to prevent excessive inflammatory responses and attacks against self. One such mechanism utilizes a subset of $CD4^+$ T cells called Tregs (Sakaguchi et al., 1995). Tregs suppress immune responses against self, altered self (tumours) and non-self antigens. Tregs can be broadly divided into two categories, natural Tregs or inducible Tregs (Sakaguchi, 2004, Wraith et al., 2004, Roncarolo et al., 2006, Costantino et al., 2008b).

### 1.2.3.1 Natural Tregs

Natural Tregs (nTregs) are thymus derived and the transcription factor forkhead box P3 (FoxP3) is critical for their development and function (Fontenot et al., 2003), with mutations of the FoxP3 gene resulting in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett et al., 2001 ) and systematic autoimmunity in the scurfy mouse (Fontenot et al., 2003). FoxP3 upregulates the expression of the IL-2 receptor alpha (CD25) and the cytotoxic T cell antigen 4 (CTLA-4) and suppresses the secretion of the cytokines IL-2, IL-4 and IFNγ (Sakaguchi et al., 2008). Paradoxically, IL-2, associated with effector cell proliferation, is an absolute requirement for nTreg development, survival and function and is believed to provide a fundamental negative feedback loop in immune regulation (Sakaguchi et al., 2008). In the mouse, $CD4^+CD25^+$ T cells identify an enriched group of nTregs. However, in humans only those cells with very 'high' CD25 expression contain an enriched group of nTregs (Baecher-Allan et al., 2001). It has become apparent that human Tregs are a more heterogeneous group than mouse Tregs and definitive markers for human nTregs still remain to be identified (Lowther and Hafler, 2012). Nonetheless, low expression of the IL-7 receptor CD127 also correlates with an enriched expression of FoxP3. CD62L (L-selectin) can be used as a marker to distinguish recently activated effector T cells from regulatory T cells, as it is downregulated after activation. Thus, an enriched population of

human nTregs can be isolated based on $CD25^{high}CD65L^{+}CD127^{low}$ expression (reviewed in (Sakaguchi et al., 2010, Lowther and Hafler, 2012)).

Although the exact mechanism of Treg suppression remains to be fully elucidated, four basic modes have been proposed. These are (1) IL-10 and TGFβ anti-inflammatory cytokine secretion, (2) effector cell cytolysis though granzyme or perforin dependent pathways, (3) metabolic disruption of effector cells, including cytokine deprivation and adenosine secretion, and (4) attenuation of APC maturation and function, including the downregulation of the costimulatory molecules CD80/CD86 (reviewed in (Sakaguchi et al., 2008, Vignali et al., 2008)). The absence of nTregs or their dysfunction is associated with a number of autoimmune diseases, such as MS (Wraith et al., 2004, Costantino et al., 2008a, Sakaguchi et al., 2010), and will be discussed in *section 1.4.3*.

#### 1.2.3.2 Inducible Tregs

In addition to nTregs that are thymic derived and matured upon exit from the thymus, naïve T cells can also be induced to become Tregs in the periphery. These Tregs are called adaptive or inducible Tregs (iTregs). Interestingly, the presence of iTregs is common at the environmental interface and their induction is promoted by factors in these regions such as Vitamin D in the skin and retinoic-acid in the gut (reviewed in (Sakaguchi et al., 2008, Vignali et al., 2008, Sakaguchi et al., 2010, Cope et al., 2011)). Three proposed subsets of iTregs have been described, $FoxP3^{+}$ iTregs, type I regulatory T cells (Tr1) and Th3 cells. $FoxP3^{+}$ regulatory iTregs can be induced upon antigen stimulation in the presence of TGFβ, IL-2 and retinoic acid (Sakaguchi et al., 2008). As FoxP3 is expressed in activated effector T cells in humans it is hypothesised that this transcription factor may play a role in facilitating a negative feedback loop in activated T cells (Sakaguchi et al., 2008, Sakaguchi et al., 2010). The latter group, Th3 cells are characterised by their large secretion of TGFβ and were first described to suppress EAE after oral administration of antigen (Chen et al., 1994). However, it

has been suggested that at least some of the Th3 subset may actually represent FoxP3$^+$ iTregs (Carrier et al., 2007). As CD46 can induce a Tr1-like phenotype and is the focus of this thesis, this subgroup is further discussed below.

#### *1.2.3.2.1 IL-10 and Tr1 T cells*

Tr1 cells are regulatory T cells characterised by the secretion of IL-10, a cytokine with potent anti-inflammatory capacity. CD46 costimulated T cells were initially described to induce a Tr1 phenotype (Kemper et al., 2003). However, this function was later shown to be defective in patients with MS (Astier et al., 2006, Martinez-Forero et al., 2008). Therefore the function of IL-10 and Tr1 cells are discussed below.

##### *IL-10*

IL-10 is a cytokine that has diverse anti-inflammatory effects on a wide range of immune cells, including T cells (reviewed in (Moore et al., 2001)). In brief, IL-10 can directly inhibit inflammatory cytokine secretion from T cells, including IL-2, TNF and IL-5. However, IL-10 primarily suppresses effector T cells through its indirect effects on professional APCs. For example, IL-10 downregulates APC's secretion of IL-1, TNF, GM-CSF and IL-12 and prevents the upregulation of costimulatory and MHC class II molecules (Moore et al., 2001). By limiting the ability of APCs to efficiently activate T cells their responses are also curtailed. In respect of this, IL-10 plays a pivotal role in preventing immunopathology (O'Garra et al., 2004, Gregori et al., 2012). For example, IL-10 knockout mice succumb to colitis and are more susceptible to EAE (Kuhn et al., 1993, Bettelli et al., 1998) and Tregs from IL-10-deficient mice were unable to suppress active EAE (Zhang et al., 2004). Moreover, suppression of EAE after treatment with a soluble myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) peptide demonstrated a key role for IL-10 in inducing tolerance (Burkhart et al., 1999, O'Neill et al., 2006, Gabrysova et al., 2009).

IL-10 is secreted by a variety of cells including several $CD4^+$ T cell subsets, B cells, dendritic cells, monocytes, macrophages, eosinophils and mast cells. Within the T cell subsets, Th2 cells were originally believed to be the primary source of IL-10 (Moore et al., 2001). However, it soon became apparent that Th1 cells could also secrete similar levels of IL-10 as Th2 cells (O'Garra and Vieira, 2007, Moore et al., 2001). It has also become evident that IL-10 secretion by Th1 cells prevents host damage by limiting excessive immune responses (Gazzinelli et al., 1996, O'Garra and Vieira, 2007, Gabrysova et al., 2009). For example, IL-10 knockout mice, infected with *Toxoplasma gondii* induced lethal effects associated with increased IFNγ, IL-12 and TNFα (Gazzinelli et al., 1996). Conversely, Th1 cells that produced elevated levels of IL-10 prevented the clearance of *Leshmania major* resulting in chronic infection (Anderson et al., 2007). Therefore the relative secretion of IL-10 and IFNγ is key to both the clearance of pathogens and preventing damage to the host. This is of particular importance to CD46 costimulated T cells as the ratio of IL-10 and IFNγ is an important factor in determining whether CD46 activated cells are regulatory or inflammatory (Cardone et al., 2010) and will be addressed in detail below.

### *Tr1*

Activation of T cells in the presence of large amounts of IL-10 can induce a subset of T cells that secrete large amounts of IL-10 and can suppress bystander effector cells (Groux et al., 1997). The first evidence of the Tr1 subset was documented in 1994 by *Bacchetta et al* who described long-term tolerance to stem cell allografts in patients with severe combined immunodeficiency (SCID). Despite the presence of host specific $CD4^+$ and $CD8^+$ T cells of donor origin there was no graft-versus-host disease (GVHD). This tolerance was believed to be the result of high levels of IL-10 and low levels of IL-2 identified in PBMCs from the patient compared to healthy controls. Indeed, host reactive $CD4^+$ T cell clones isolated from the SCID patient also expressed high levels of IL-10 and low

levels of IL-2 upon antigen stimulation *in vitro* (Bacchetta et al., 1994). *Groux et al* further investigated these suppressive IL-10 secreting T cells and determined that antigen specific $CD4^+$ T cells that undergo chronic stimulation in the presence of IL-10 proliferate poorly, produce high amounts of IL-10 and IL-5, secrete moderate levels of IFNγ and TGFβ, low levels of IL-2 and no IL-4. These antigen specific T cells could suppress autologous effector T cells partially through the effects of IL-10 and TGFβ secretion *in vitro* and *in vivo* in an antigen dependent manner (Groux et al., 1997). Moreover, IL-10 acts as a positive autocrine factor in the induction of IL-10 secreting cells, which is in contrast to its effects on both Th1 and Th2 development (Barrat et al., 2002). Importantly, co-transfer of these antigen specific T cells with pathogenic $CD4^+CD45RB^{high}$ T cells could suppress colitis induced in SCID mice (Groux et al., 1997). More recently, islet specific IL-10 secreting Tregs have also been isolated from non-diabetic individuals (Tree et al., 2010). Interestingly, pathogen specific Tr1 cells were also found in the respiratory tract of mice infected with *Bordetella pertussis.* It is believed that these Tr1 cells are induced by the bacteria to avoid clearance by Th1 immune responses. This highlights how bacteria have evolved to manipulate Tr1 cells in order to evade host immune system detection (McGuirk et al., 2002*)*.

Although the secretion of IL-10 is the main mechanism of Tr1 function, Tr1 cells also employ contact dependent suppression mechanisms, which are *reviewed in* (Gregori et al., 2012). In brief, the inhibitory co-receptors CTLA-4 and PD-1 play a key role in the suppression of allergen specific T cells. Similar to $FoxP3^+$ Tregs, Tr1 cells also induce metabolic disruption in effector T cells through the production of adenosine (Mandapathil et al., 2010) (Gregori et al., 2012). Furthermore, Tr1 cells can kill target cells through the expression of granzyme B (Grossman et al., 2004). Tr1 cells are similar to nTregs in their low proliferative capacity, minimal IL-2 secretion, cell-cell contact suppression (Sakaguchi et al., 2008). Importantly however, Tr1 cells do not express high levels of FoxP3, the transcription factor that defines nTregs and $FoxP3^+$ iTreg (Vieira et al., 2004) suggesting that they are separate lineages.

*In vitro differentiation of Tr1 cells*

*Groux et al* originally demonstrated that antigen specific T cells activated by APCs in the presence of IL-10 induced a Tr1 phenotype (Groux et al., 1997). However, IL-10 was not sufficient to induce Tr1 cells in the absence of professional APCs suggesting that other soluble factors or costimulatory molecules were involved in their induction. In this regard, it was noted that the addition of IFNα alongside IL-10 markedly increased differentiation of naïve T cells into Tr1 cells (Levings et al., 2001). Further investigations also reported that the immunosuppressive drug, Rapamycin, also enhanced IL-10 induction of Tr1 T cells and induced a state of long term tolerance in diabetic mice after pancreatic islet transplantation (Battaglia et al., 2006). Of note, treatment of naïve T cells with a combination of dexamethasone, vitamin D and neutralising antibodies against IL-4, IFNγ and IL-12 produced a more homogenous population of IL-10 producing cells that did not express IL-4, IL-5 or IFNγ. In accordance with their cytokine secretion profile, the expression of T-bet and GATA-3 traditionally associated with Th1 and Th2 subsets, was also downregulated (Barrat et al., 2002). IL-27, a member of the IL-12 cytokine family can also induce a Tr1 regulatory cell phenotype while concomitantly inhibiting Th17 differentiation (reviewed in (Pot et al., 2011a)). In this regard, IL-27 was shown to play a protective role in EAE models (Fitzgerald et al., 2007) and is also upregulated in patients with MS that positively respond to interferon-β (IFNβ) treatment (Sweeney et al., 2011). In addition to soluble factors in the milieu, Tr1 cells can also be induced by APCs. For example, repeated exposure of naïve T cells with both immature and IL-10 induced tolerogenic dendritic cells can induce Tr1 cells with suppressive capacity (Levings et al., 2005, Gregori et al., 2010). Moreover, tolerogenic dendritic cells can induce Tr1 cells after only one round of stimulation (Gregori et al., 2010) suggesting that chronic stimulation may not be an absolute requirement of Tr1 induction. The fact that in the absence of APCs, IL-10 alone is not sufficient to induce Tr1s suggests that costimulatory molecules could play an important role. Indeed, in an airway hyper reactivity (AHR) model, dendritic cells induced Tr1 cells in an ICOS-ICOS ligand dependent fashion. After adoptive transfer these Tr1 cells inhibited AHR in sensitised mice (Akbari

et al., 2002). Interestingly, IL-27 induced Tr1 cells also upregulated the ICOS receptor which acted to boost Tr1 differentiation (Pot et al., 2009). This suggests that the ICOS pathway may play an important role in Tr1 development. Other costimulatory molecules can also induce Tr1 cells including CD2 and CD46 (Wakkach et al., 2001, Kemper et al., 2003). Indeed, CD2 costimulated naive T cells also required only one stimulation to induce a suppressive Tr1 phenotype (Wakkach et al., 2001). The role of CD46 induced Tr1s is central to this thesis and is discussed in *section 1.3*.

Importantly, it remains to be determined if Tr1 cells are generated from naïve cells or are simply a terminal differentiation state of other T cell subsets, such as Th1 or Th2 subsets (Cope et al., 2011, Pot et al., 2011b). In light of this, the existence of Tr1 T cells as a distinct subtype of T cells remains controversial. Nonetheless, the uniting characteristic of all Tr1 cells or IL-10 producing Tregs is that they produce high amounts of IL-10 and can suppress bystander T cell responses (O'Garra et al., 2004, Gregori et al., 2012). Identification of Tr1 specific markers will help resolve the identity or Tr1s as unique T cell subset or terminal differentiation state of other T helper subsets (Pot et al., 2011b).

CD46 costimulated T cells can induce either a Tr1-like or Th1-like phenotype (Kemper et al., 2003, Sanchez et al., 2004, Cardone et al., 2010). In MS patients, CD46 induced IL-10 secretion was defective (Astier et al., 2006, Martinez-Forero et al., 2008). Given the importance of IL-10 and Tregs in suppressing inflammation, the role of CD46 in regulating T cell activation was investigated. The structure and known functions of CD46 are described below. Both the structure of CD46 and its previously described functions give useful insights into how CD46 might regulate T cells' phenotype.

# 1.3 CD46

## 1.3.1 Background

CD46 is a type I transmembrane protein that is expressed in all nucleated cells except erythrocytes (*figure 1.3*) (Seya et al., 1986). CD46 is not expressed in the mouse, except in the testis (Tsujimura et al., 1998). CD46 has the ability to regulate T cell (Astier et al., 2000, Kemper et al., 2003, Oliaro et al., 2006, Ni Choileain and Astier, 2011), B cell (Fuchs et al., 2009), monocyte (Karp et al., 1996), macrophage (Hirano et al., 1999, Kurita-Taniguchi et al., 2000, Katayama et al., 2000, Kurita-Taniguchi et al., 2002), dendritic (Barchet et al., 2006, Vaknin-Dembinsky et al., 2008), astrocytes (Ghali and Schneider-Schaulies, 1998) and epithelial cell (Kallstrom et al., 1998, Lee et al., 2002, Ludford-Menting et al., 2002, Cardone et al., 2011) responses during inflammation. CD46 influences various cell functions including cytokine secretion (Karp et al., 1996, Ghali and Schneider-Schaulies, 1998, Kemper et al., 2003), cell activation (Astier et al., 2000), apoptosis/necrosis (Elward et al., 2005, Cole et al., 2006, Grumelli et al., 2011), polarity (Ludford-Menting et al., 2002, Oliaro et al., 2006, Ludford-Menting et al., 2011), morphology (Zaffran et al., 2001), migration (Alford et al., 2008, Tsai et al., 2012), autophagy (Joubert et al., 2009), antigen presentation (Rivailler et al., 1998), toll-like receptor activation (Iacobelli-Martinez and Nemerow, 2007), wound repair (Cardone et al., 2011) and has more recently been shown to have an anti-microbial function (Basmarke-Wehelie et al., 2011). Moreover, dysregulation of CD46 responses have been reported in several immunopathologies including MS (Astier et al., 2006, Martinez-Forero et al., 2008, Ma et al., 2009), systematic lupus erythematosus (SLE) (Le Buanec et al., 2011), asthma (Xu et al., 2010, Tsai et al., 2012), rheumatoid arthritis (RA) (Cardone et al., 2010), atypical haemolytic uremic syndrome (HUS) (Richards et al., 2003) and human C3 deficiency (Ghannam et al., 2008). Despite its role in such a wide range of immune responses much of CD46 intracellular signalling pathways remains to be elucidated.

### 1.3.2 CD46 Structure

The basic structure of CD46 consists of an extracellular domain of four short consensus repeats (SCR1-4), three exons rich in serine, theronine and proline (STP A, B and C), a region of unknown significance, a transmembrane segment, and a short intracellular tail (*figure 1.3*) (Liszewski et al., 1991).

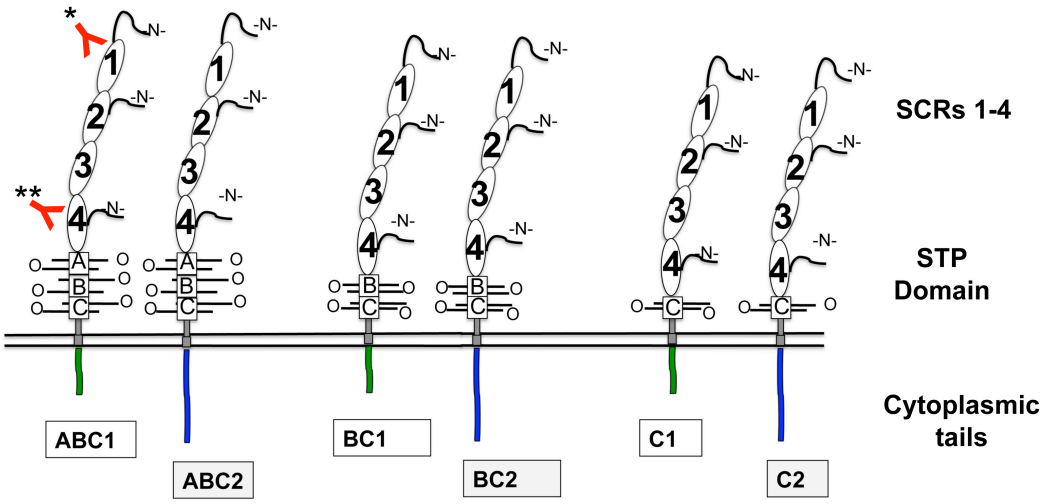


***Figure 1.3: Schematic of Cyt1 and Cyt2 isoforms with STP splicing.*** *Schematic of six different CD46 isoforms as a result of splicing of the STP and cytoplasmic regions – ABC1, ABC2, BC1, BC2, C1, and C2. Antibody binding sites used in this study are denoted with asterisk: * MCI20.6 and E4.3, **MEM-258.*

### 1.3.3 Isoform Splicing

At the DNA level, CD46 consists of 14 exons and multiple isoforms of CD46 are produced as a result of alternative mRNA splicing (Russell et al., 1992). Splicing of exon 13 gives rise to two distinct cytoplasmic tails: Cyt1 and Cyt2 (*figure 1.4*) (Liszewski et al., 1991, Russell et al., 1992). Cyt1 and Cyt2 isoforms are co-expressed in all cell types. However, there is preferential expression for Cyt2 in the brain and kidney (Johnstone et al., 1993). In addition, the STP region can also be alternatively spliced resulting in the isoforms BC-Cyt1 (BC1), BC-Cyt2 (BC2), C-Cyt1 (C1) or C-Cyt2 (C2) (*figure 1.3*) (Seya et al., 1999). Individuals express both the BC and C isoforms of CD46, however, the exact ratio of BC:C expression varies. Dominant expression of the BC isoform is most common

(65%), and dominant expression of the C isoform is the least common (6%), the remainder of the population have equal expression of both isoforms (Ballard et al., 1987). Tissue specific expression of the STP-ABC, BC and C isoforms also exists (Seya et al., 1999). For example, the ABC isoform is primarily expressed in the intestine, salivary gland and cancer cells (Xing et al., 1994). The BC isoforms are preferentially expressed in the kidney and foetal heart, while the C isoform is preferentially expressed in the brain (Johnstone et al., 1993). This suggests that there is a biologically significant role for distinct CD46 proteins. Importantly for this project, in a transgenic mouse model expressing Cyt1- or Cyt2-CD46, these isoforms had contrasting roles in regulating a contact hypersensitivity reaction (*discussed below*) (Marie et al., 2002). Prior to beginning this investigation, their function or expression during human T cell activation had not been reported.

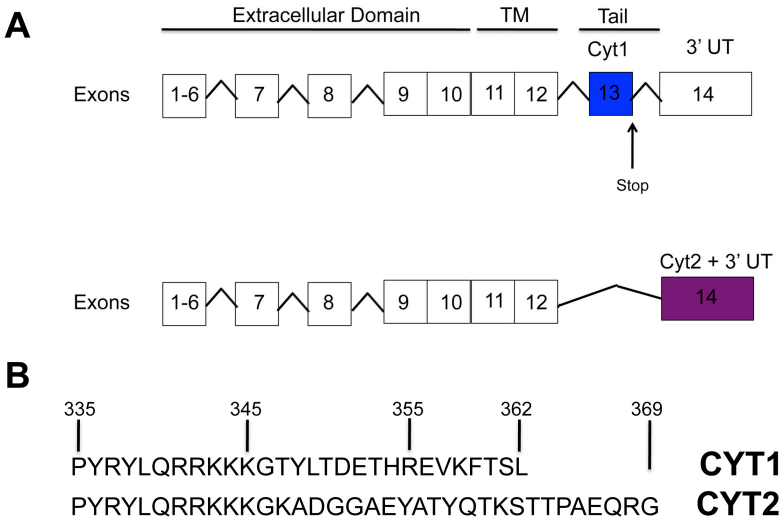


**Figure 1.4: Schematic of CD46 exons, cytoplasmic tail splicing and amino acid sequences.** ***(A)*** *CD46 Exons: (Exon 1) 5' untranslated region (UT), (Exon 2-6) SCRs, (Exon 7-9) STP domain, (Exon 10) Unknown, (Exon 11-12) TM – transmembrane domain.* **(***Exon 13) Cyt1 and also a stop codon (indicated by arrow). (Exon 14) Cyt2. If exon 13 is translated (blue) exon 14 is converted into the 3' untranslated region (UT). If exon 13 is absent then exon 14 (purple) encodes the cyt2 tail and the 3' UT region.* ***(B)*** *Amino acid sequence of Cyt1 and Cyt2: This schematic is adapted from "Weyand NJ, Lee SW, Higashi DL et al Monoclonal antibody detection of cd46 clustering beneath Neisseria gonorrhoeae microcolonies. Infect Immun. 2006".*

## 1.3.4 CD46 Glycosylation

### 1.3.4.1 Background

Glycosylation of proteins is a post-translational modification and, similarly to phosphorylation, it is an important regulator of receptor cell signalling (Ohtsubo and Marth, 2006). Due to its complexity, however, it is often overlooked. In contrast to the nucleic acids of amino acids monosaccharides are not limited to a linear assembly and can form much more complex 3D structures (Varki, 2009, Clark and Baum, 2012b). Thus, glycans give a huge diversity to a limited number of amino acids and support the huge complexities associated with biological systems such as the immune system. Throughout T cell development in the thymus and in the periphery, global cell surface changes in glycosylation occur (Piller et al., 1988, Galvan et al., 1998, Comelli et al., 2006, Clark and Baum, 2012a), which can alter T cell activation (Demetriou et al., 2001, Lau et al., 2007, Chen et al., 2007, Chen et al., 2009), migration (Sperandio et al., 2009) and apoptosis (Galvan et al., 2000, Nguyen et al., 2001, Hernandez et al., 2006). Such changes in glycosylation are largely the result of alterations of golgi transferase expression levels, which are highly sensitive to differentiation state, extracellular cytokine milieu (Wagers et al., 1998, Grabie et al., 2002, Toscano et al., 2007) and genetic variations (Mkhikian et al., 2011). Glycan alterations can initiate subtle or large immunological responses by regulating protein stability (Tifft et al., 1992, Pulido and Sanchez-Madrid, 1992), localisation (Brewer et al., 2002, Chen et al., 2007), ligand affinity (Demetriou et al., 2001, Lau et al., 2007) and subsequent cell signalling (Demetriou et al., 2001, Lau et al., 2007, Chen et al., 2009). Indeed, modulations of transferases are associated with regulatory and inflammatory T cell phenotypes (Wagers et al., 1998, Jenner et al., 2006, Toscano et al., 2007), autoimmunity (Demetriou et al., 2001, Mkhikian et al., 2011) and cancer (Partridge et al., 2004). There are two commonly studied types of glycans, N-glycans and O-glycans, both of which have attachment sites on CD46. To date, there have been no studies addressing the role of CD46's N- or O-glycans during T cell activation. However, in *Chapter 5* the role of these glycans in regulating CD46 costimulation will be described. Although no studies have

investigated the structure of CD46's glycans in depth, the general structure of N- and O- glycans is described below.

#### 1.3.4.2 N-glycans

N-glycosylation of CD46 occurs on SCR1, SCR3 and SCR4 (*figure 1.3*)(Lublin et al., 1988). N-glycosylation refers to the covalent binding of a sugar to the nitrogen of asparagine with an Asn-X-Ser/Thr amino acid sequence, where X is any amino acid except proline. N-glycosylation occurs as the nascent protein passes through the endoplasmic reticulum (ER) and Golgi (Ohtsubo and Marth, 2006, Varki, 2009, Clark and Baum, 2012b). The majority of N-glycans have the same core structure ($Man_9GlcNAc_2$) and three subtypes exist which are classified by the sugars that are attached to the core; high mannose, hybrid and complex. High mannose glycans are the initial glycans attached to proteins but their sugars can be trimmed by glycosidases, giving access to glycotransferases to add non mannose sugars thereby creating hybrid or complex glycans (*figure 1.5*) (Ohtsubo and Marth, 2006, Varki, 2009, Clark and Baum, 2012b). Complex N-glycans contain lactosamine repeats that are important for lectin binding, cellular interactions and migration. This core group can then be modified by the addition of more sugars like sialic acid, fructose or acetyl groups. This is termed 'branching' or 'decorating'. All three N-glycan subtypes are present on T cells (Ohtsubo and Marth, 2006, Clark and Baum, 2012b). With respect to CD46's function, N-glycans were shown to support measles virus (MV) and *Neisseria* infection (Maisner et al., 1994, Maisner et al., 1996, Hsu et al., 1997, Kallstrom et al., 2001) and also complement inactivation (Liszewski et al., 1998). The glycans are believed to play a role in regulating the correct conformation of CD46 for ligand binding (Persson et al., 2010). Glycosylation changes in CD46 could therefore affect ligand binding and conformation changes during T cell activation, potentially regulating its function.

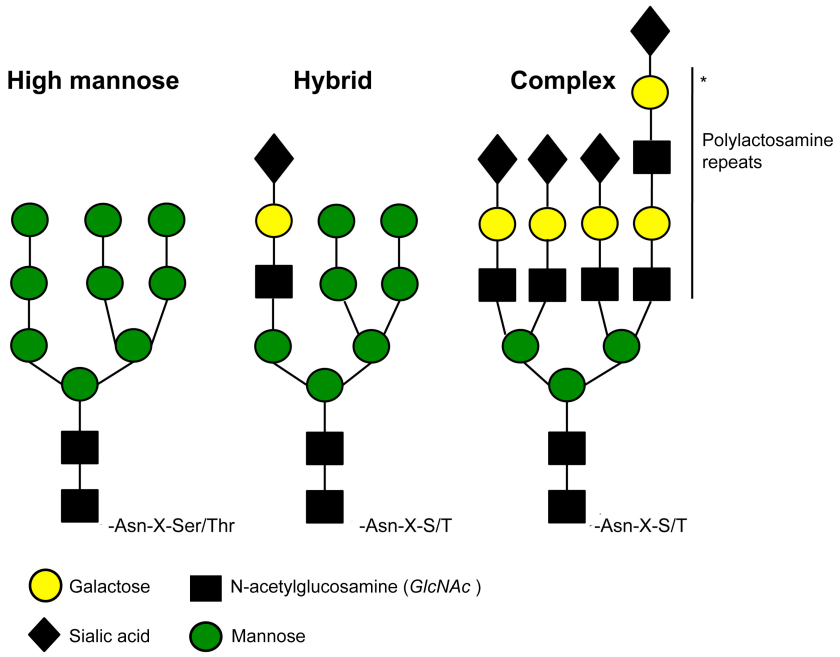


***Figure 1.5: Schematic of N-glycans subsets.*** *High mannose, hybrid and complex. This schematic is adapted from "Clark MC, Baum LG. T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. Ann N Y Acad Sci. 2012".*

#### 1.3.4.3 O-glycans

O-glycosylation of CD46 occurs in the STP region (*figure 1.3*). STP splicing regulates the potential number of O-glycan sites. The STP-A contains five potential O-glycosylation binding sites, the STP-B contains ten (Xing et al., 1994) and the STP-C region contains 2 sites (http://www.uniprot.org/uniprot/P15529, accessed 09/02/12). O-glycosylation refers to the binding of a glycan to the hydroxyl group of serine and theronine and generally occurs in the Golgi apparatus. O-glycosylation occurs serially, where the first sugar added to serine or theronine proteins is N-acetylgalactosamine (GalNAc). Extension of the first sugar results in the formation of different core O-glycan groups. Addition of the first sugar, GalNAc results in the formation of the Core 1 structure (asialo core 1). If sialic acid is added to the core structure it is known as sialo core 1. Extension of this sugar with N-acetylgalactosamine (GlcNAc) forms the Core 2 structure (*figure 1.6*) (Ohtsubo and Marth, 2006, Clark and Baum, 2012a). Similar to N-glycans, Core

2 structures can be further extended to form lactosamine repeats. Core 1 and Core 2 structures are commonly expressed on T cells and are altered during T cell activation (Varki, 2009, Clark and Baum, 2012b). Although research into the glycosylation of CD46 is limited, earlier studies have identified that both the mature forms contain sialic acid, complex N-glycans and O-glycans (Yu et al., 1986, Ballard et al., 1988). Interestingly, the more heavily glycosylation BC isoform has an increased ability to bind and deactivate C4b providing enhanced protection to the classical complement pathway (Liszewski and Atkinson, 1996). This suggests that glycosylation may be important for regulating CD46 function. The effects of CD46 glycosylation during T cell activation were investigated during this project and are described in *Chapter 5*.

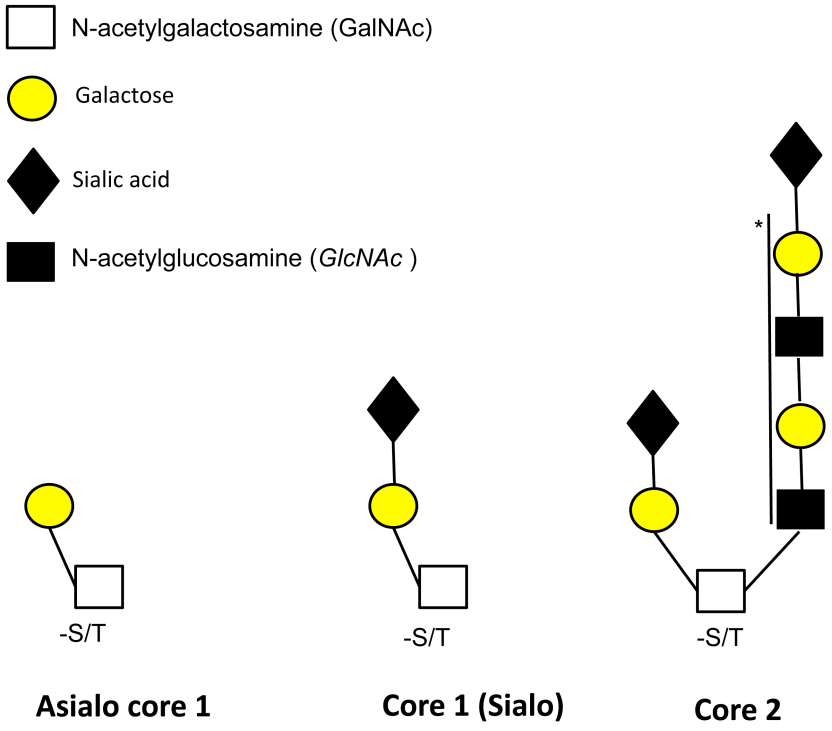


***Figure 1.6: Schematic of O-glycan subsets.*** *Asialo core 1, Sialo core 1 and core 2 o-glycans. The line and asterix represents a polylactosamine repeat. This schematic is adapted from "Clark MC, Baum LG. T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. Ann N Y Acad Sci. 2012".*

### 1.3.5 Functions

The role of CD46 during T cell activation is the basis of this project. Initial studies of CD46 function in the literature are focused on the role of CD46 as a complement regulator and as a pathogen receptor. For descriptive purposes, CD46's functions are therefore categorised into three groups: complement regulator, T cell costimulatory molecule and a pathogen receptor (*figure 1.7*). However, these functions are not mutually exclusive and CD46's function in T cell activation is intrinsically linked to its other roles (Ni Choileain and Astier, 2011, Ni Choileain and Astier, 2012). Understanding the functions of CD46 in other capacities gives important clues as to how CD46 can regulate T cell phenotypes. Each function is described below. In the case of complement regulation and the pathogen receptor functions, their relationship with CD46's role as a costimulatory molecule is also discussed.

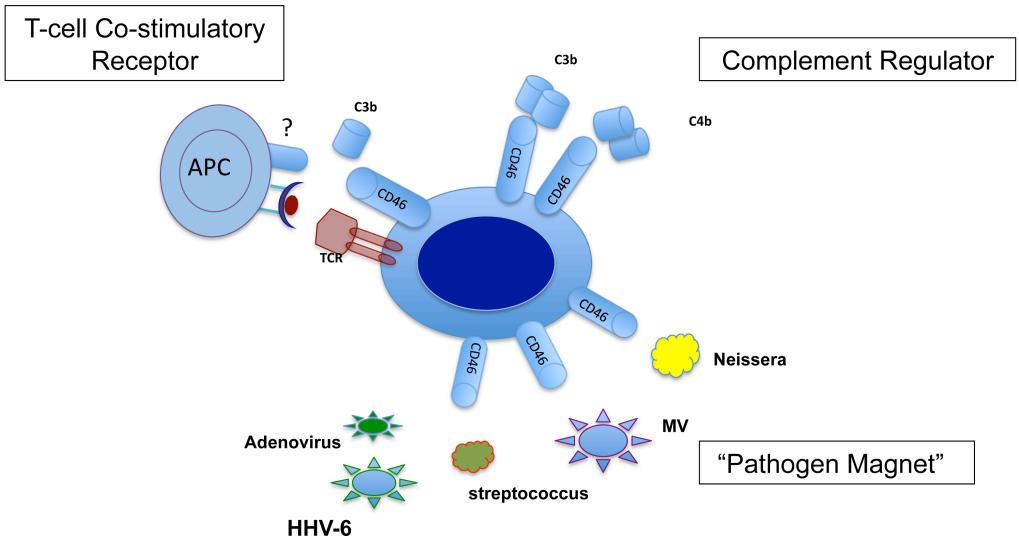


***Figure 1.7: A schematic of the functions of CD46 in immunology.***

#### 1.3.5.1 T cell activation

Over a decade ago CD46 was identified as a T cell co-stimulatory molecule that linked the innate and adaptive immune systems. *Astier et al* demonstrated that

CD46 costimulation in $CD4^+$ T cells induced marked actin relocalisation, morphological changes, strong proliferation and involved the activation of the T cell signalling molecules $p120^{CBL}$, LAT, Vav, Rac, Erk and MAPK (Astier et al., 2000, Zaffran et al., 2001). Following on from these reports, CD46 costimulation in the presence IL-2 was shown to induce the secretion of large amounts of IL-10 with little or no IL-2, IL-4 or IL-5 (Kemper et al., 2003, Price et al., 2005). Due to the cytokine profile of these T cells and their ability to suppress effector T cells, CD46 activated T cells were termed Tr1 T cells (Kemper et al., 2003, Price et al., 2005). Several reports expanded on the role of CD46 activated T cells as regulatory T cells. For example, CD46-activated Tr1 cells express granzyme B and can kill target cells in a perforin dependent manner (Grossman et al., 2004). Moreover, non-identified soluble factors released by CD46-activated T cells were also shown to play a role inhibiting *Mycobacterium bovis* BCG specific $CD4^+$, $CD8^+$ and γδ T cell responses. Of note IL-10 played no role in the suppression of γδT cells highlighting the ability of CD46 to suppress inflammation through other mechanisms (Cardone et al., 2010). Thus, CD46 activated T cells can induce different regulatory functions in an inflammatory environment. Of note, factors that promote the IL-10-induced differentiation of Tr1 cells also support the differentiation of CD46-Tr1 cells. For example, IFNα also been shown to skew nTreg differentiation towards Tr1 differentiation in the presence of CD46 ligation. Moreover, active lupus is related to increased levels of complement stimulation of CD46 and high levels of IFNα that inhibit nTreg contact dependent suppression (Le Buanec et al., 2011). Furthermore STAT3 which is fundamental to Tr1's role in inducing IL-10 secretion (Gregori et al., 2012) binds to the CD46 promoter and induces its upregulation (Buettner et al., 2007). Indeed, CD46 costimulation resulted in increased levels of phosphorylated STAT3 (pSTAT3), suggesting a positive feedback loop. In correlation with these findings, CD46 costimulation of T cells in the presence of the immunomodulator prostaglandin E2 (PGE-2), induced decreased levels of pSTAT3, that correlated with a reduced Tr1 phenotype (Kickler et al., 2012). Moreover, although CD46-Tr1 cells have been reported to have a strong proliferative capacity, further studies have noted that with suboptimal TCR stimulation, CD46-Tr1 cells display a reduced

proliferative capacity, reminiscent of other Tregs, that was the result of a defective Akt/Survivin pathway (Meiffren et al., 2006). This study reflected the observations made by *Adams et al* that adenovirus infection or CD46 crosslinking in the presence of low CD3 stimulation induced reduced T cell proliferation (Adams et al., 2012).

Despite these findings questions remain over the exact phenotype of CD46 induced T cells. *Sanchez et al* demonstrated that CD46 costimulation in the presence of PMA or CD28 stimulation increased IFNγ and IL-2 secretion, characteristic of a Th1 phenotype (Sanchez et al., 2004). Later it was proposed that CD46 Tregs originate from a Th1 subset. The concentration of IL-2 was deemed crucial for switching CD46 activated T cells from a Th1 to an IL-10 producing Treg phenotype. During early activation CD46 induces IFNγ secretion, however as the concentration of IL-2 increases CD46 activated cells begin to switch to IL-10 secreting cells (Cardone et al., 2010). Notably, CD46 regulatory cells are now termed Tr1-like cells to reflect their potential Th1 origins, cytokine profile and their suppressive function. Regardless of the origins of CD46 Tregs, their cytokine profile remains distinct to that of classic CD28 costimulated T cells as they display increased ratio of IL-10:IFNγ and have low levels of IL-2 secretion (Kemper et al., 2003, Kickler et al., 2012). Importantly, CD46 induced IL-10 secretion was defective in MS patients and CD28 induced IL-10 secretion was not (Astier et al., 2006). In summary, CD46 activation can alter the phenotype of T cells by promoting a suppressive phenotype. Further investigations are required, however, to determine the exact phenotype of CD46 costimulated T cells. Of note, however, the plasticity of CD46 responses may be explained by the diversity of its cytoplasmic tails. As mentioned, a key study by *Marie et al* using a contact hypersensitivity model in a transgenic mouse model expressing either Cyt1- or Cyt2-CD46 demonstrated that Cyt1 suppressed inflammation and Cyt2 promoted inflammation (Marie et al., 2002). Prior to commencing this project, the role of Cyt1 and Cyt2 in human T cell activation had not been addressed. Therefore, the expression levels and functions of Cyt1

and Cyt2 during human T cell activation were hypothesised to play a role in regulating CD46 function and were investigated in *Chapter 3* and *Chapter 4*.

#### 1.3.5.2 Complement regulator

Over two decades ago, CD46 was identified as a member of the regulators of complement activation (RCA) family gene cluster, located on chromosome 1, 1 q32 (Lublin et al., 1988). CD46 protects autologous cells from complement attack by acting as a cofactor for the factor-1 mediated cleavage of C3b and C4b (Seya et al., 1986, Seya et al., 1999, Liszewski et al., 1991). Binding of complement requires the SCR2-4 domains (Adams et al., 1991, Seya et al., 1998). Atypical HUS is a pathology characterised by massive complement activation on renal endothelial cells, thrombocytopenia, microangiopathic haemolytic anaemia and acute renal failure (Kavanagh et al., 2006). Several mutations in CD46 that affect complement binding or cleavage predispose individuals to atypical HUS (Richards et al., 2003). Furthermore, expression of CD46 in the female reproductive tract fluid is also believed to protect the foetus from complement attack (McLaughlin et al., 1996). Moreover, certain tumor cells upregulate CD46 expression and release soluble CD46 to evade complement attack and enhance metastasis (Gorter and Meri, 1999, Hakulinen et al., 2004, Tuve et al., 2006), therefore, CD46 expression regulates susceptibility to complement attack. Moreover, C3b ligation of CD46 in the presence of TCR stimulation induces a Tr1-like phenotype in T cells (Kemper et al., 2003). The expression levels of cell surface CD46 during T cell activation could therefore regulate complement induced cell death and CD46 induced T cell responses. Prior to this study, surface expression of CD46 during T cell activation had not been examined and is detailed in *Chapter 3*.

#### 1.3.5.3 Pathogen Receptor

CD46 was labelled a ‘pathogen magnet’ due to the large number of bacteria and viruses that it binds (Cattaneo, 2004). CD46’s function as a pathogen receptor

was established when it was found to bind to the Edmonton strain of MV and was named gp57/67 due to its molecular weight (Dorig et al., 1993, Naniche et al., 1993a). Today, CD46 has been identified as the receptor for several group B adenoviruses (Segerman et al., 2003, Gaggar et al., 2003), herpes virus 6 (HHV-6) (Santoro et al., 1999), streptococcus (Okada et al., 1995) and pathogenic *Neisseria* (Kallstrom et al., 1997). Pathogen ligation of CD46 can induce cellular responses that can aid both host defense and pathogen survival. In defense of the host, CD46 pathogen binding can induce inflammatory cytokine production (Ghali and Schneider-Schaulies, 1998), antibody class switching (Imani et al., 1999), autophagy (Joubert et al., 2009) and increased sensitivity to complement attack (Schnorr et al., 1995). Conversely, CD46 can also facilitate a pathogen's adhesion and entry into cells and activate signalling pathways that regulate cytokine production (Karp et al., 1996, Price et al., 2005) and function (Oliaro et al., 2006) in their favour. Indeed, the popularity of CD46 with pathogens emphasizes the importance of CD46 signalling in regulating the immune response. How a cell's function is regulated by pathogen ligation of CD46 is described below.

#### *1.3.5.3.1 Measles Virus*

Measles virus (MV) is a negative strand RNA virus that infects the respiratory tract (Gerlier et al., 1994a). Immune suppression follows MV infection and is associated with a high mortality rate and a marked decrease in IL-12 production by monocytes (Karp et al., 1996). MV binds to CD46 SCR1-2 domains, which facilitates cell entry and replication of the virus (Dorig et al., 1993, Naniche et al., 1993a, Manchester et al., 1995, Iwata et al., 1995, Greenstone et al., 2002). *In vitro* CD46 activation in monocytes by MV or C3b induces a decrease in IL-12 production (Karp et al., 1996). Therefore, CD46 signalling was thought to play a key role in MV-induced immunosuppression and introduced evidence that pathogens could manipulate CD46 signalling pathways to regulate cytokine production. However, MV ligation of CD46 in macrophages supports host defence by increasing IL-12, IFN-α/β and nitric oxide production thereby

promoting macrophage activation and reducing viral replication (Hirano et al., 1999, Kurita-Taniguchi et al., 2000, Katayama et al., 2000). Differences in the maturation state of monocytes are believed to regulate CD46's role in regulating IL-12 secretion. Notably, however, CD46's regulation of IFN-α/β and nitric oxide required its cytoplasmic tail (Hirano et al., 1999, Katayama et al., 2000) and indicated that active signalling was involved in altering cell function. To date, expression levels of Cyt1 and Cyt2 in monocytes and macrophages have not been analysed. However, as Cyt1 and Cyt2 have contrasting roles in regulating inflammation (Marie et al., 2002), differences in their expression could explain its apparently contrasting responses during MV infection.

MV activation of CD46 in astrocytes and B cells also affected their function (Ghali and Schneider-Schaulies, 1998, Imani et al., 1999). Specifically, CD46 crosslinking on astrocytes or treatment with MV proteins can induce IL-6 production (Ghali and Schneider-Schaulies, 1998) and MV infection or CD46 crosslinking prior to IL-4 treatment of B cells induced IgE class switching (Imani et al., 1999). Of note, IL-6 secreting B cells were recently shown to play a role in promoting disease in the EAE model (Barr et al., 2012). Whether CD46 crosslinking in B cells can also induce increased IL-6 secretion may be an interesting avenue of research. These data indicate that CD46 can induce signalling in a variety of cell types that influence the immune response. Understanding the expression and regulation of CD46 in T cells will be important starting point to an overall understanding of CD46's role in the immune response.

#### *1.3.5.3.2 Human Herpes Virus*

HHV-6 is a double stranded DNA virus that is present in the majority of the humans without major complications and preferentially infects $CD4^+$ T cells (De Bolle et al., 2005). HHV-6 is normally latent but under periods of immune weakness its reactivation has been associated with direct immunosuppression and serious opportunistic infections (De Bolle et al., 2005). Interestingly, due to its

latency, periodic reactivation and neurotropism, HHV-6 has also been linked to MS, albeit controversially (Clark, 2004). MS relapses and EDSS score have been linked to viral replication and increased levels of HHV-6 in cerebral plaques and serum (Berti et al., 2002, Cermelli et al., 2003, Alvarez-Lafuente et al., 2006), supporting the hypothesis that HHV-6 reactivation is linked to MS. Cross-reactive epitopes have also been identified on HHV-6 and myelin basic protein (MBP) and therefore T cells could potentially become activated by HHV-6 in the periphery and trigger an autoimmune response to myelin in the central nervous system (CNS) via a process known as 'molecular mimicry' (Tejada-Simon et al., 2003, Cheng et al., 2012). HHV-6 adherence to CD46 was dependent on the SCR2 and SCR3 domains (Greenstone et al., 2002). Upon binding to CD46 HHV-6 causes the relocation of CD46 to lipid rafts allowing the entry of HHV-6 into the cell via pH dependent cell fusion (Tang et al., 2008). *Ludford-Menting et al* have shown that the localisation of CD46 into lipid rafts plays a role in regulating CD46 function in T cells (*discussed in Chapter 4*) (Ludford-Menting et al., 2011) and suggests that HHV-6 could induce CD46 relocation to lipid rafts as a mechanism to induce specific signalling. Furthermore, CD46 expression on astrocytes and oligodendrocytes is believed to support cell to cell fusion with infected $CD4^+$ T cells aiding the transmission of the virus into the CNS (Tang et al., 2008). Therefore, CD46 could facilitate the spread of pathogens with myelin cross-reactive epitopes into the CNS. Moreover, the presence of HHV-6 in the CNS may provide additional local signalling ligands for CD46. These findings underline the importance of understanding CD46 signalling pathways, which likely have important consequences for regulating the immune response in the CNS.

#### *1.3.5.3.3 Adenovirus*

Human adenoviruses are double stranded DNA viruses that are classified into different subgroups (A-F) (Wang et al., 2007). Several viruses in the subgroup B utilize CD46 as their receptor including Adenovirus type 35 (Ad35). Ad35 binds to CD46 via SCR1 and SCR2 and is mainly associated with urinary tract and

kidney infection (Tuve et al., 2006, Wang et al., 2007). Recent studies highlight the ability of adenoviruses to manipulate the adaptive immune system. Specifically, adenovirus infection of dendritic cells induces high levels of IFNα and cell maturation that is CD46 dependent (Lore et al., 2007) suggesting that CD46 signalling acts to protect the host upon adenovirus infections. However, IFNα has also been shown to promote CD46 Treg function (Le Buanec et al., 2011) and therefore may also act as negative feedback loop that regulates the inflammatory response. Moreover, Ad35 can inhibit $CD4^+$ T cell IL-2 secretion predominantly through its knob protein actions with CD46 (Adams et al., 2011, Adams et al., 2012). Direct treatment of $CD4^+$ T cells with Ad35 or antibody cross-linking of CD46 also decreased naïve $CD4^+$ T cell proliferation and inhibited $NF_kB$ activation in CD28 costimulated cells (Adams et al., 2011). Decreased T cell proliferation upon Ad35 ligation of CD46 reflects observations by *Meiffren et al* who identified abortive proliferation in CD46 activated T cells when low doses of TCR stimulation were used (Meiffren et al., 2006). Reduced/low proliferation of T cells upon CD46 costimulation is in contrast to previous reports that have shown that CD46 ligation induced increased T cell proliferation (Astier et al., 2000, Ni Choileain et al., 2011). The differences in these reports likely reflect lower levels of TCR stimulation and raise questions that the strength of TCR ligation may regulate CD46's function. In *Chapter 5*, the role of the TCR in regulating CD46 expression and structure is elucidated. To conclude, the varying roles of CD46 in regulating immune cell responses upon pathogen ligation suggest that CD46 regulation is complex and that different mechanisms are in place, including TCR stimulation, to regulate CD46's function.

#### *1.3.5.3.4 Neisseria*

*Neisseria* is a gram-negative bacterium that utilizes the surface organelle type IV to bind CD46 and adhere to cells. The pili of both *Neisseria gonorrhoeae* and *Neisseria meningitides* bind to CD46 (Kallstrom et al., 1997). *Kallstrom et al* demonstrated that the SCR3, STP region that the cytoplasmic tails also plays an

active role in bacterial adherence (Kallstrom et al., 2001). Moreover, upon ligation, Cyt2 becomes tyrosine phosphorylated and this involves the Src kinase c-Yes (Lee et al., 2002). Antibody ligation of CD46 in Jurkat T cells also induces the tyrosine phosphorylation of Cyt2 by Lck, an Src kinase (Wang et al., 2000). Therefore, bacterial binding to CD46 initiates active signalling that supports the bacterial infection. Again, *Neisseria* ligation of CD46 emphasizes the ability of pathogens to manipulate CD46 signalling, for their advantage.

#### *1.3.5.3.5 Streptococcus pyogenes*

*Streptococcus pyogenes* is a gram-positive bacterium that causes group A streptococcal infection that ranges from minor skin infections to deeper infection in the fascia causing life threatening necrotizing fasciitis. The streptococcus M protein binds to CD46 facilitating attachment to the cell and evasion of oponization by the alternative complement pathway (Okada et al., 1995, Rezcallah et al., 2005). CD46 transgenic mice become highly susceptible to necrotizing fasciitis upon infection leading to death (Matsui et al., 2009). Bacterium adherence requires the SCR3 and SCR4 domains and the cytoplasmic tails were shown to play a role in the bacterium's entry into epithelial cells (Giannakis et al., 2002, Rezcallah et al., 2005). Moreover, upon CD46 ligation the streptococcal M protein can manipulate the adaptive immune system by inducing T cells to secrete large amounts of IL-10 and granzyme B production (Price et al., 2005). This reflects the finding of *Mcguirk et al* who demonstrate that antigen specific Tr1s were induced by *Bordetella pertussis* in an attempt to avoid Th1 responses (McGuirk et al., 2002). *Streptococcus* also induced shedding of cell surface CD46 from epithelial cells (Lovkvist et al., 2008). Interestingly, pathogen manipulation of CD46's signalling capacities are often associated with its downregulation: shedding or internalisation. In fact, all of the pathogens described above induce CD46 downregulation (Naniche et al., 1993b, Gill et al., 2003, Sakurai et al., 2007, Lovkvist et al., 2008). Considering the role of these pathogens in regulating CD46 signalling, it raises the question of whether CD46 downregulation or shedding is important for CD46 costimulation. Prior to this

report, the downregulation of CD46 cell surface expression in human primary T cells had not been investigated. Previous reports have described CD46 downregulation in other cell types and these are described below.

### 1.3.6 CD46 expression and downregulation

It is known that CD46 can be downregulated from the cell surface as a result of internalisation and/or surface shedding (Naniche et al., 1993b, Van Den Berg et al., 2002, Gill et al., 2003, Crimeen-Irwin et al., 2003, Hakulinen et al., 2004, Elward et al., 2005, Sakurai et al., 2007, Lovkvist et al., 2008, Weyand et al., Hakulinen and Keski-Oja, 2006), however its downregulation in primary human T cells had not been specifically investigated. In order to determine if downregulation is important for CD46 signalling, its method of downregulation needs to be addressed during T cell activation and is described in *Chapter 3*. *Crimeen-Irwin et al* elucidated the mechanism of CD46 internalisation in lymphoid and non-lymphoid cells using either monovalent or multivalent antibody ligation. Upon monovalent ligation of CD46 in non-lymphoid cells, including monocyte and dendritic cells, CD46 was constitutively internalised after 30 min via clathrin coated pits to multivesicular bodies, in the vicinity of the Golgi and recycled to the cell membrane. Internalisation of CD46 into clathrin-coated pits was dependent on the interaction of its YRYL amino acid sequence and the clathrin adaptor protein AP-2. Constitutive internalisation was not observed in lymphoid cells at this timepoint and was therefore believed to act as a sensor for APCs and support antigen presentation (Crimeen-Irwin et al., 2003). Indeed, expression and downregulation of CD46 facilitates highly efficient antigen presentation of MV particles by the MHC Class II molecule in murine B cells (Gerlier et al., 1994b). Upon CD46 multivalent crosslinking in both lymphoid and non-lymphoid cells, CD46 is degraded following downregulation through macropinocytosis. Of note, the cellular pool of CD46 is thought to be in dynamic equilibrium with the cell surface and multivesicular bodies (MVB). Therefore any CD46 in MVB is rapidly sent to the surface where it can be internalised and degraded. It is hypothesised that CD46 may be prevented from

recycling in lymphoid cells by the binding of $p56^{lck}$ as is the case for CD4 (Sleckman et al., 1992).

Shedding of CD46 from the surface membrane can be the result of the release of the full intact form or a soluble form that does not contain the cytoplasmic tail. The full length CD46 is shed from various apoptotic cancer cell lines including the Jurkat T cell line (Hakulinen et al., 2004, Elward et al., 2005). Increased shedding of CD46 was associated with increased deposition of C3b on apoptotic cells indicating how CD46 can regulate "don't eat me" signals (Elward et al., 2005). Chemically induced apoptosis enhanced sCD46 levels which were due to cleavage of CD46 by metalloproteinases (MMP)(Hakulinen et al., 2004). Furthermore, the venom from Loxoceles and *Neisseria* infection induces cleavage of CD46 from epithelial cells (Van Den Berg et al., 2002, Weyand et al.). Further studies have identified specific MMPs involved in CD46 proteolysis. CD46 is cleaved by ADAM10 during apoptosis of epithelial cells (Hakulinen and Keski-Oja, 2006) and MMP3, -8, -9 during neuronal cell apoptosis (Cole et al., 2006). Of note both the vesicular CD46 and the sCD46 retain their ability to mediate cleavage of C3b by factor I (Hakulinen et al., 2004). The release of functionally competent CD46 into the milieu may act to taper complement activation in the surrounding area and act as a negative feedback loop under inflammatory conditions.

In summary, CD46 downregulation occurs by different routes and so far has been identified in the involvement of pathogen entry, complement regulation, apoptosis and antigen presentation. Prior to this study it remained to be determined how CD46 was specifically downregulated in primary human T cells and if downregulation occurred, what was its purpose? Understanding how CD46 expression is regulated and how this affects its functions in T cells will advance our understanding of the possible mechanisms of CD46 dysfunction in MS, RA and asthma. Defective IL-10 secretion upon CD46 costimulation was first

described in patients with MS (Astier et al., 2006, Astier and Hafler, 2007). It was these findings that initiated the investigation of CD46 expression and function in this project.

## 1.4 Multiple Sclerosis

MS is a chronic inflammatory disease of the CNS characterised by multifocal demyelination and progressive neurodegeneration (Sospedra and Martin, 2005, Zozulya and Wiendl, 2008, Petermann and Korn, 2011, Nylander and Hafler, 2012). Clinical symptoms vary depending on the location of the lesions but commonly include chronic and relapsing paralysis, sensory dysfunction and cognitive impairment. Although MS is believed to be a heterogeneous disease, disease types are commonly classified into three subtypes. The most common form of MS is relapsing-remitting MS (RRMS) (85-90%) and involves periodic subacute clinical events followed by complete or almost complete recovery. However, over time the majority of patients with RRMS will eventually develop irreversible progressive neurological damage termed secondary progressive MS (SPMS). The third subtype is called primary progressive MS (PPMS), which is a more aggressive form of the disease. PPMS occurs in about 10-15% of cases and follows a progressive disease course in the absence of the relapsing –remitting phase of the disease (Sospedra and Martin, 2005, Zozulya and Wiendl, 2008, Petermann and Korn, 2011, Nylander and Hafler, 2012).

### 1.4.1 Causes

MS is an autoimmune disease that is believed to occur due to a combination of genetic and environmental factors (Sospedra and Martin, 2005, Goverman, 2011, Nylander and Hafler, 2012). The role of genetics in contributing to disease was established early on and demonstrated that the probability of developing the disease was 20-50 fold higher in first-degree family members than unrelated individuals. Furthermore, in monozygotic twins, the probability of a second twin

having MS is roughly 25% (Sospedra and Martin, 2005). To date, variations in the MHC class II genes is associated with strongest independent risk factor in MS. Moreover, with the onset of the genome wide association studies (GWAS) over the last five years, large strides in the identification of specific risk factor genes have taken place. The GWAS studies analysed the whole genome with the aim of identifying genetic variations associated with disease. Of note there is strong enrichment of identified genes associated with lymphocytes and particularly T cell function and proliferation. For example, susceptibility to disease has been linked with the cytokine pathway (IL2R, IL7R, IL7, IL12), signal transduction (STAT3, STAT4, T-bet) and costimulatory molecules (CD58, CD80, CD86, CD40) (Sawcer et al., 2011b). However, genetics do not account for the entirety of MS susceptibility and in the last few years there has been intense research into the role of sunlight exposure and Vitamin D levels in MS (Cantorna et al., 1996, Correale et al., 2009, Ascherio et al., 2010, Sawcer et al., 2011b). There is a strong correlation with latitude and the incidence of MS, whereby countries further north with less sunshine have increased incidence of MS. In line with this, evidence indicates that Vitamin D deficiency is associated with an increased incidence of disease (Ascherio et al., 2010). Moreover active vitamin D has been shown to have immunomodulatory properties including Treg induction and the attenuation of EAE severity (Correale et al., 2009, Cantorna et al., 1996). Importantly, genetic risk factors associated with Vitamin D metabolism have also been identified (Sawcer et al., 2011b) highlighting how environmental and genetic factors could combine to increase disease susceptibility. Other environmental factors include Epstein–Barr virus (EBV) infection and this is evidenced by the fact that 100% of MS patients have EBV compared to 90% of healthy people (Ascherio and Munger, 2007). It has been demonstrated that EBV specific T cells cross react with myelin antigens and raise the possibility that EBV could enhance risk susceptibility as a result of ‘molecular mimicry’ (Lang et al., 2002, Lunemann et al., 2008).

It is clear from our current understanding that the immune system plays a key role in the etiology of MS. In accordance with this and extensive research in EAE (Sospedra and Martin, 2005, O'Connor and Anderton, 2008, Wraith and Nicholson, 2012, Nylander and Hafler, 2012), a model was developed to explain the initiation of the inflammatory cascade in MS that leads to the neurological damage. It is hypothesised that autoreactive T cells to myelin become activated in the periphery and migrate across the blood brain barrier (BBB). Once within the CNS, these T cell become reactivated by local APCs and initiate a cascade of events that lead to myelin destruction (Goverman, 2011) (*figure 1.8*). Curiously, autoreactive T cells to myelin components are present within both healthy individuals and patients with MS (Sun et al., 1991, Zhang et al., 1994, Raddassi et al., 2011). Therefore, determining why these myelin reactive T cells in patients become activated and not in healthy controls has been intensively investigated (Costantino et al., 2008a, Goverman, 2011, Nylander and Hafler, 2012). Two main concepts are thought to be involved: (1) there is a break in tolerance that allows these reactive cells to become activated; and (2) infectious agents such as EBV activate myelin specific T cells as a result of molecular mimicry (Goverman, 2011). As Tregs play a pivotal role in maintaining immunological homeostasis, examining their development and function in MS patients for potential discrepancies became a key focus of MS research. The role of Tregs in MS is discussed below.

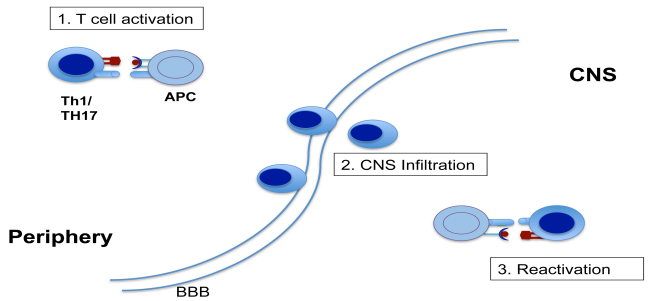


***Figure 1.8: Model of T cell induced multiple sclerosis. Myelin autoreactive** T cells are activated in the periphery. Following activation they migrate across the blood brain barrier (BBB) into the central nervous system (CNS) where they become reactivated. Reactivation of T cells initiates the inflammatory cascade that leads to myelin damage and the symptoms of MS.*

### 1.4.2 $CD4^+$ T cells and MS

$CD4^+$ T cells can cause direct damage to neurons through the secretion of pro-inflammatory cytokines or through orchestrating other immune cells such as B cells, macrophages and NK cells to cause direct damage to neurons via autoantibody secretion, phagocytosis and cytolysis respectively (Venken et al., 2010). Indeed, animal models of MS, such as EAE, highlighted a central role for the involvement of $CD4^+$ T cells. In genetically susceptible mice, immunization with myelin proteins in the presence of adjuvant or myelin reactive $CD4^+$ T cells was sufficient to induce disease with a pathology similar to that observed in MS (O'Connor and Anderton, 2008, Sospedra and Martin, 2005). Moreover, an increased frequency in MOG reactive T cells were found in MS patients compared to healthy controls (Zhang et al., 1994, Raddassi et al., 2011). As mentioned previously, genetic studies have identified the MHC class II DRB1 gene as the strongest association to MS susceptibility (Sawcer et al., 2011a), these data strongly associated $CD4^+$ T cells with the pathogenesis of MS. With respect to patient treatments, Alemtuzumab, an anti-CD52 monoclonal antibody, that reduces T cell numbers, significantly improves RRMS disability after 6 months treatment (Coles et al., 2006). Thus evidence from the EAE model, genetic studies and MS treatment support the proposal that T cells are key players in driving disease (Nylander and Hafler, 2012). The subtype of T helper cells responsible for driving EAE was originally believed to be the Th1 subset and was evidenced by the protective effect of T-bet knockdown and IL-12 neutralisation in mice with EAE (Leonard et al., 1995, Bettelli et al., 2004). Furthermore treatment of MS patients with IFNγ, the trademark Th1 cytokine, was shown to exacerbate disease (Panitch et al., 1987). However, knockout of key Th1 differentiation molecules, $IFN\gamma^{-/-}$, $IFN\gamma R^{-/-}$ and $IL\text{-}12p35^{-/-}$ (a subunit of the IL-12p35/p40 heterodimer), in mice failed to protect them from EAE and increased disease susceptibility (Ferber et al., 1996, Willenborg et al., 1996, Becher et al., 2002). It was then discovered that the IL-12 subunit p40 was not only shared with p35 but also p19 to form IL-23, which we know now to be involved in Th17 cell differentiation and EAE susceptibility (Oppmann et al., 2000, Cua et al., 2003). Indeed, both subtypes are now accepted to play an important role in EAE

induction (Petermann and Korn, 2011). The balance of Th1 and Th17 that are involved in inducing disease likely varies within patients and could be an important variable in treatment of the disease (Wraith and Nicholson, 2012). More research is required to determine the exact role of Th1 and Th17 subsets in MS.

Although, there is no doubt that $CD4^+$ T cells play a role in MS pathology there is increasing evidence that other immune cells such as $CD8^+$ T cells and B cells are also involved. For example, the predominant T cell presence in lesions is that of $CD8^+$ T cells. Furthermore, clonal expansion of $CD8^+$ T cells has been identified in lesions and axonal damage is closely linked to their activity (Neumann et al., 2002). B cells also appear to play an important role as demonstrated by the consistent presence of increased immunoglobulin levels and clonally expanded B cells in the CSF of MS patients' (Franciotta et al., 2008). As mentioned, depletion of B cells also ameliorates disease in the EAE model (Barr et al., 2012). Moreover, Rituximab an anti-CD20 monoclonal antibody that is used to treat MS selectively depletes B cells, thereby reducing lesions and relapses in patients (Nylander and Hafler, 2012). Taken together, these findings indicate that MS is an autoimmune disease, in which myelin reactive $CD4^+$ T cells, in addition to other adaptive immune cells, play a fundamental role.

### 1.4.3 Tregs

As describer earlier, Tregs are crucial in regulating inflammatory responses and their function is defective in MS (Viglietta et al., 2004, Wraith et al., 2004, Astier and Hafler, 2007, Costantino et al., 2008a, Venken et al., 2010). The role of nTregs in EAE has been extensively studied and their ability to regulate disease susceptibility and severity has been investigated in depletion and co-transfer models (reviewed in (O'Connor and Anderton, 2008)). Importantly, *McGeachy et al* demonstrated that the depletion of nTregs inhibited the natural resolution of EAE (McGeachy et al., 2005) and emphasised the importance of nTregs in

disease recovery. Moreover, FoxP3$^+$ iTregs could also block EAE in an IL-10 dependent manner (Selvaraj and Geiger, 2008), thereby underscoring the potency of both Treg subsets and IL-10 in regulating disease susceptibility. Initial studies of CD4$^+$CD25$^{high}$ Tregs from MS patients demonstrated that patients have similar levels of CD4$^+$CD25$^{high}$ Tregs as healthy controls (Viglietta et al., 2004). However, CD4$^+$CD25$^{high}$ Tregs in RRMS were shown to be functionally defective in the suppression of CD4$^+$CD25$^-$ effector cells (Haas et al., 2005, Viglietta et al., 2004). The defect of MS Tregs could be attributed to a lower frequency of FoxP3 that is reduced in the CD4$^+$CD25$^{high}$ Treg population and at the single cell level (Venken et al., 2008). Further examination of the Treg subset by *Michel et al* demonstrated that it was the CD127$^{high}$ subset of CD4$^+$CD25$^{high}$ cells that were defective in MS. CD4$^+$CD25$^{high}$CD127$^{high}$ cells were hyperproliferative and produced increased levels of pro-inflammatory cytokines IFNγ, TNFα and IL-2 in MS patients compared to healthy controls (Michel et al., 2008). Recently, the extent of Treg plasticity has come to the forefront and it is recognised that Tregs are also capable of secreting inflammatory cytokines (Zhou et al., 2009). Dominguez-Villar et al have identified an increased frequency of Th1-like FoxP3$^+$ Treg (CD4$^+$CD25$^{high}$CD127$^{low/-}$) in RRMS patients compared to healthy controls. This population was characterised by IFNγ secretion, T-bet expression and reduced suppressive capacity. Moreover, this population is sensitive to IFNβ therapy which restores their frequency to that of healthy controls (Dominguez-Villar et al., 2011). Treg deficiency is not only evident in the periperphy but also appears to occur during their development in the thymus. For example, studies investigating recent thymic emigrating Tregs (RTE-Tregs) showed a decreased frequency of these cells in the periphery of RRMS patients compared to healthy controls (Haas et al., 2007) (Venken et al., 2008). These developments indicate that MS patients have defective Treg development in the thymus and a disturbed Treg homeostasis.

### 1.4.4 CD46 dysregulation in MS

As discussed earlier, CD46 is a T cell co-stimulatory molecule (Astier et al., 2000) that induces a Tr1-like phenotype characterised by the secretion of large amounts of IL-10 (Kemper et al., 2003). However, in MS patients CD46 stimulated T cells produced little or no IL-10 in comparison to healthy controls. This defect was specific to CD46 stimulated T cells and was not observed upon CD28 costimulation (Astier et al., 2006). Of note, higher stimulation of CD3 or increased concentrations of IL-2 did not restore IL-10 secretion (Astier et al., 2006, Martinez-Forero et al., 2008). This study was reproduced by two independent reports, one using an *in vivo* MS monkey model (Martinez-Forero et al., 2008, Ma et al., 2009). Furthermore defects in CD46 induced IL-10 secretion have also been documented in asthma (Xu et al., 2010, Tsai et al., 2012) and RA (Cardone et al., 2010). The mechanism for this defect remains to be elucidated. Using a transgenic mouse model expressing either the Cyt1 or Cyt2 isoform of CD46. *Marie et al* demonstrated that Cyt1 attenuated and Cyt2 promotes inflammation in a contact hypersensitivity model (Marie et al., 2002). Interestingly, *Astier et al* also reported increased expression of Cyt2 mRNA in MS patients suggesting that differences in CD46 isoform expression may contribute to the inflammatory response in MS (Astier et al., 2006). CD46 costimulated T cells from asthma patients also expressed increased levels of Cyt2 and decreased levels of Cyt1 at the RNA level (Tsai et al., 2012). Therefore there is evidence to suggest that the expression levels of the Cyt1 and Cyt2 isoforms may play a role in determining CD46 induced IL-10 production.

In summary, CD46 has wide-ranging signalling capacities in a variety of cell types that intertwine both the innate and adaptive immune responses. These functions are utilised by pathogens to alter the inflammatory response in their favour. CD46's ubiquitous expression and the breadth of its functional duties suggest that CD46 signalling pathways are complex. Expression of different CD46 isoforms, namely Cyt1 and Cyt2, and the glycosylation of CD46 likely facilitates specific responses in different environments and inflammatory

conditions. Specifically of interest to this project is the role of CD46 in inducing regulatory T cells, which is characterised by the secretion of IL-10. This function is defective in MS patients and was the reason for initiating this project. In order to understand why IL-10 secretion was defective in MS patients, the expression, regulation and function of CD46 was investigated in healthy controls (*Chapters 3-5*). In *Chapter 6,* the findings from healthy controls were compared to those from patients with MS. Indeed, defects in CD46 expression and regulation were noted in MS patients and provide an important platform for understanding defective IL-10 production in T cells from these patients. Furthermore, given the wider context of CD46 function in the immune system, understanding the regulation of CD46 in T cells will no doubt have implications for other cell types and the regulation of the immune system as a whole.

## 1.5 Working Hypothesis

1. CD46 expression is regulated by T cell activation.
2. The cytoplasmic tails, Cyt1 and Cyt2, have different functions.
3. Altered expression of CD46 is responsible for the defect of CD46 upon activation in MS.

## 1.6 Aims

1. To determine the expression levels of surface CD46, intracellular Cyt1 and Cyt2 upon T cell activation in healthy controls.
2. To determine the function of the Cyt1 and Cyt2 isoforms during T cell activation.
3. To determine the mechanism(s) of the regulation of expression of CD46 in T cells.
4. To identify defects in CD46 expression/regulation that may account for a decrease in IL-10 production in MS patients.

# Chapter 2 : Material and Methods

## 2.1 General Reagents

All chemical reagents were sourced from Sigma-Aldrich unless otherwise stated.

## 2.2 Blood donors

To assess CD46 expression and function in healthy controls and patients with MS, $CD4^+$ T cells were isolated from peripheral blood. Blood samples were taken by venupuncture into heparin vacutainers (Sarstedt, Germany). The characteristics of the healthy and patient donors are described in *table 2.1*. Blood donations were taken after informed consent was given. Ethical approval was obtained from the Lothian Board Ethics Committee prior to obtaining blood donations from patients.

***Table 2.1: Characteristics of the donors used in this study.***

| | **Healthy Controls** | **Patients with MS** |
|---|---|---|
| **Sex (Female:Male)** | 1:1 | 3.6:1 |
| **Age (year, mean ± SD)** | 35.1±7.9 | 42.5±9.5 |
| **Age range** | 25-55 | 26-58 |
| **EDSS (mean ± SD)** | - | 2.9±1.6 |
| **Treatment** | - | 10 untreated/13 IFNβ |

# 2.3 $CD4^+$ T cell isolation

## 2.3.1 Isolation of peripheral blood mononuclear cells

In order to isolate $CD4^+$ T cells, peripheral blood mononuclear cells (PBMC) were first isolated from whole blood using density gradient centrifugation (Hettich Rotina 420). Heparinised whole human blood was diluted 1:2 in RPMI 1640 medium (PAA, GE Healthcare, UK) at room temperature under sterile conditions. The diluted blood sample was gently layered above the Ficoll-paque plus (GE Healthcare, UK) in 50 ml Falcon tubes (15 ml Ficoll and 35 ml diluted blood) to produce two separated phases. The tubes were then centrifuged for 25 min at 990 x g without brakes. This resulted in the formation of a cloudy mononuclear cell layer, which was removed from the interphase carefully with a Pasteur pipette and transferred into a new 50 ml Falcon tube. The cells were then washed four times with Phosphate buffered saline (PBS) (PAA, GE Healthcare, UK) at 460 x g for 10 mins. Finally an aliquot of the purified cells was diluted with trypan blue and counted using a counting chamber.

## 2.3.2 CD4+ T cell Isolation

$CD4^+$ T cells were negatively isolated from PBMCs using magnetic beads ($CD4^+$ Isolation Kit II; Miltenyi Biotec, Auburn, USA). PBMCs and reagents were kept on ice at all times in sterile culture conditions. The PBMCs were resuspended in 40 µl MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) per $10^7$ total cells. The biotin-antibody cocktail was added at 10 µl per $10^7$ total cells. Then the cells were mixed well and incubated on ice for 10 mins. Following the incubation, 30 µl of MACS buffer and 20 µl of anti-biotin microbeads per $10^7$ total cells was added and mixed well. Cells were incubated on ice for 15 mins, and then washed with 15 mls of MACS buffer at 370 x g for 5 mins. After centrifugation, cells were resuspended in 500 µl of MACS buffer and the $CD4^+$ cells were isolated by magnetic separation using a large size (LS) column (Miltenyi Biotec, Auburn, USA) and a midiMACS™ separator (Miltenyi Biotec, Auburn, USA). The LS column was placed in the magnetic field of the separator and was rinsed with 3

mls of MACS buffer. The cell suspension was gently placed at the bottom of the column and allowed to pass through into a 50 ml Falcon tube. The column was then washed three times with MACS buffer. The entire effluent was collected in the same 50 ml Falcon tube. This tube now contained the enriched population of unlabelled $CD4^+$ T cells. $CD4^+$ T cell purity was assessed by flow cytometry using a mouse αCD4-fluorescein isothiocyanate (FITC) monoclonal antibody (clone OKT4, eBioscience, San Diego, USA) and the $CD4^+$ T cell isolations with a purity of >90% were used for further experiments.

## 2.4 T cell activation

In order to activate $CD4^+$ T cells, tissue culture plates (Nunc, Rochester, NY) were pre-coated with αCD3 (clone OKT3, 5 μg/ml), αCD28 (5 μg/ml) or αCD46 (clone MC120.6, 10 μg/ml), kindly provided by Dr. Chantal Rabourdin-Combe from Lyon in France. $CD4^+$ T cells were stimulated with αCD3 alone, αCD3/CD28, αCD3/CD46 or αCD3/CD28/CD46. When T cells were transfected with the CD19-fusion proteins, cells were also activated with the mouse αCD19 monoclonal antibody at a concentration of 10 μg/ml (clone MCA19; AbD Serotec, UK). In one experiment the natural ligand of CD28, recombinant B7-1 (R&D Systems, USA) was used to stimulate the T cells at a concentration of 2.5 μg/ml. Recombinant human IL-2 (10 U/ml, Roche, Switzerland) was added to αCD3/CD46 stimulated cells to induce a regulatory phenotype as previously described (Kemper et al., 2003, Astier et al., 2006). The concentration of activating antibodies were as indicated, unless otherwise stated, and were diluted in PBS. The cell culture plates were coated with the antibody dilutions for 2 hrs at room temperature then washed once with PBS. $CD4^+$ T cells were seeded onto to the plates at the required density, suspended in 10% RPMI 1640 (10% v/v foetal calf serum (FCS)(Bio Sera, East Sussex, UK), 1% minimum essential medium non essential amino acids (MEM NEAA) 100X (PAA, GE Healthcare, UK), 1% v/v sodium pyruvate (PAA, GE Healthcare, UK), 1% v/v HEPES 1M (PAA, GE Healthcare, UK), 1% v/v penicillin streptomycin 100x (PAA, GE Healthcare, UK), 1% v/v L-glutamine 200 mM 100x (PAA, GE Healthcare,

UK)). Cells were activated for a period of up to 5 days. In two experiments phorbol myristate acetate (PMA) (5 ng/ml) and ionomycin (50 ng/ml) were used to activate T cells. In these experiments, T cells were plated in uncoated 48 well culture plates in 10 % RPMI 1640 and PMA/Ionomycin was added to the medium. The cells were cultured overnight and then harvested for flow cytometry analysis to assess CD46 and CD69 expression, described in *section 2.7*.

## 2.5 Protein and pathway inhibition

### 2.5.1 Protease Inhibition

In some experiments the protease inhibitors of MMP/ADAM or PγS were added to $CD4^+$ T cell cultures at the time of activation. The MMP/ADAM inhibitor was GM6001 (Merck Chemicals, Germany) and was added to T cell cultures at a concentration of 10 μM. The PγS inhibitor used was DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)] -S-phenylglycinet-Butyl Ester) and was added at a final concentration of 10 μM.

### 2.5.2 Protein Degradation

In order to inhibit the endosomal/lysosomal pathway $CD4^+$ T cells were pre-incubated overnight at 37 $^oC$ with chloroquine (at 10 μg/ml unless otherwise stated). Likewise, inhibition of proteosome degradation was achieved by pre-incubating $CD4^+$ T cells overnight at 37 $^oC$ with MG132 (Boston Biochem, Cambridge USA) at the indicated concentrations. Following incubation with the inhibitors, T cells were activated as previously described.

### 2.5.3 Glycosylation

In some experiments, inhibitors of N- and O- glycosylation were used. In order to inhibit N-glycosylation $CD4^+$ T cells were pre-incubated overnight with tunicamycin (15 μg/ml). To inhibit O-glycosylation, $CD4^+$ T cells were pre-

incubated overnight with Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (Benzyl-α-GalNAc) (5 mM). After incubation the cells were activated as previously described.

# 2.6 Transfection

## 2.6.1 CTF Constructs

The cytoplasmic transmembrane fragment (CTF) constructs were kindly provided by our collaborators Dr. Nathan Weyand and Prof. Maggie So (University of Arizona, Tucson, USA). The wild-type Cyt1 and Cyt2 CTFs (CTF1 and CTF2) and their mutant counterparts (UNCLF.1 and UNCLF.2), that could not be cleaved by PγS were created as previously described (Ni Choileain et al., 2011) and cloned into the pIRESneo vectors (GenBank Accession no. U89673). In order to construct the uncleavable UNCLF.1 and UNCLF.2, the predicted PγS cleavage site in the transmembrane domain of the wild-type CTF (CTF wt) was mutated (CTF VV_GG) as shown in *figure 2.1A*. The cleavage site was predicted based on the known PγS cleavage site for Notch (*figure 2.1A)*. The mutation successfully inhibited the cleavage of the CTF by PγS in CHO cells and is demonstrated by the absence of the ~6 kDa PγS cleavage product (*figure 2.1B)*.

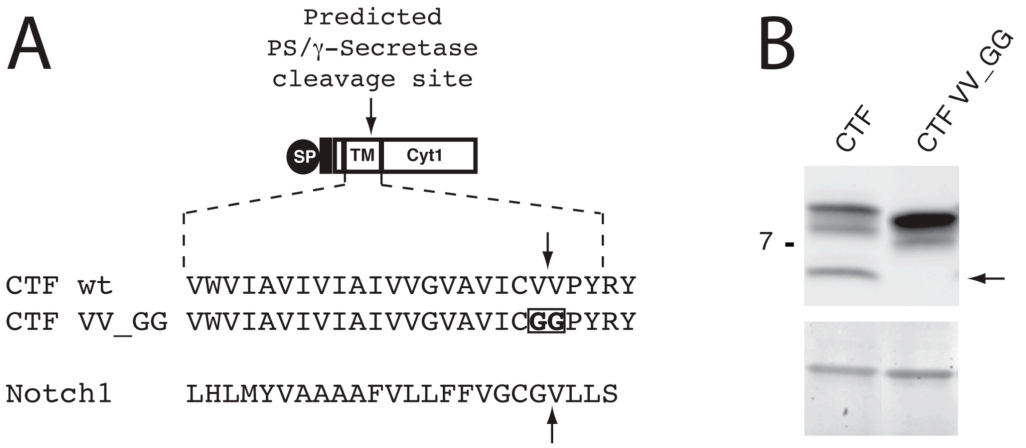


***Figure 2.1: Mutation of the CTF constructs. (A)*** *(Top diagram) Schematic of the Cyt1 cytoplasmic transmembrane fragment (CTF) (CTF1) construct and the predicted PγS cleavage site is indicated with a downward arrow. SP = heterologous signal peptide, TM= transmembrane domain, Cyt1 = Cyt1 cytoplasmic tail (the Cyt2 cytoplasmic tail was used for the CTF2 constructs). (Bottom diagram) The amino acid sequences of the wild-type (CTF wt) and the mutated (CTF VV_GG) CTF proteins. The PγS cleavage site is indicated with the downward arrow and the mutated amino acids are boxed. As a comparison, the cleavage site of Notch1 is shown with an upward arrow.* ***(B)*** *Expression of the wt and mutant CTFs in CHO cells. The arrow indicates the ~6 kda peptide released upon PγS cleavage. GAPDH was used as a loading control.*

### 2.6.2 Fusion proteins

The CD19 fusion proteins were constructed by fusing the extracellular domain of CD19 to either the Cyt1 or Cyt2 CTFs (CTF1 and CTF2) of CD46. These fusion proteins were called CD19-Cyt1 and CD19-Cyt2 and were kindly provided by Joelle Thomas, Université Lyon, France. The protein fragments were obtained from HeLa and Raji cell lines, fused and cloned into the pcDNA3 vector (Invitrogen) as previously described (Ni Choileain et al., 2011). Sequencing was used to verify the correct construction of all chimeric proteins.

### 2.6.3 Amaxa cell transfection

Amaxa nucleofection (Lonza, Switzerland) was carried out according the manufacturers guidelines. Briefly, prior to transfecting T cells, culture medium

(10% RPMI 1640) was pre-warmed at 37 $^{o}$C in a 48 well plate. Freshly isolated T cells (~5 x $10^{6}$ per transfection condition) were transferred into a 1.5 ml eppendorf and washed once with PBS. The PBS was removed and the cell pellet was gently resuspended by flicking the tube. Then 1 μg of the required plasmid was added to the cell suspension, followed by 100 μl of the nucleofactor reagent. After mixing gently, the cell suspension was transferred to a cuvette, avoiding bubble formation and placed in the AMAXA nucleofection device, which was set to the program U-14. Following transfection, using a Pasteur pipette, a little of the pre-warmed culture medium from the 48 well plate was added to the cuvette and the transfected cells were transferred to the 48 well plate. The T cells were allowed to rest overnight at 37 $^{o}$C. The following day, the transfected T cells were activated as normal.

### 2.6.4 Fugene cell transfection

For the experiments using the CD19 fusion proteins, HEK-293 cells were transfected with the FuGENE HD transfection Reagent Kit (Roche, Switzerland). HEK-293 were cultured overnight at 37 $^{o}$C in a 6 well plate at 2.5 x $10^{5}$/ml (per transfection condition) in 10% RPMI 1640. The FuGENE HD transfection reagent was preincubated with 1 μg of the required plasmid for 15 mins at room temperature and then added to the required well. The cells were cultured overnight and the following day they were assessed for CD19, Cyt1 and Cyt2 expression by flow cytometry.

## 2.7 Flow cytometry

### 2.7.1 Cell surface staining

The cell surface expression of CD46, CD69, CD25, CTLA-4, CXCR3 and CXCR4 were determined using fluorescent-labelled antibodies specific for these receptors. After the required T cell activation period, ~1 x $10^{5}$ T cells were harvested from each activating condition. Of note, for cell surface staining, $CD4^{+}$

T cells and reagents were kept on ice at all times. The T cells were span down at 13000 RPM for 20 sec and washed twice with fluorescence-activated cell sorting (FACS) buffer (2% v/v FCS in PBS). After washing, the cells were incubated on ice for 20 mins, in the dark, with the required fluorescent-labelled antibodies diluted in FACS buffer. The antibodies for flow cytometry were purchased from BD Pharmingen (San Jose, USA) unless otherwise stated and were as follows: anti-CD46-FITC (clone MEM-258, Biolegend, San Diego, CA, USA), anti-CD69-PE (clone FN50), anti-CD25-Allophycocyanin (APC) (clone M-A251), and anti-CTLA-4-phycoerythrin (PE) (clone BNI3), anti-CD19-PE (clone HIB19), anti-CXCR3-FITC (R&D Systems, Minneapolis, USA), anti-CXCR4-PE (R&D Systems, Minneapolis, USA). In addition, T cells from each activated condition were also incubated with their corresponding isotype controls in order to determine the level of background fluorescence. The isotypes used were purchased from BD Pharmingen. After incubation, the cells were washed 3 times with FACS buffer and resuspended in 200 µl FACS buffer. The cells were then analysed for their cell surface expression of CD46, CD69, CD25, CTLA-4, CXCR3 and/or CXCR4 using the BD Biosciences FACSCalibur flow cytometer equipped with Cell Quest software. $CD4^+$ cells were gated based on the forward and side scatter to exclude debris and dead cells. The data was exported and then analysed using FlowJo 8.8.4 software (TreeStar USA).

### 2.7.2 Intracellular staining

In order to determine the expression levels of Cyt1 and Cyt2 upon T cell activation, cytoplasmic tail-specific monoclonal antibodies were used, these were designed by our collaborators Dr. Nathan Weyand and Prof. Maggie So (University of Arizona, Tucson, USA) (Weyand et al., 2006). After the indicated T cell activation period, ~2 x $10^5$ T cells were harvested from each activating condition. The T cells were span down at 11000 x g for 20 sec and washed twice with FACS buffer (2% v/v FCS in PBS). After washing, the cells were incubated at room temperature for 20 mins with either the anti-Cyt1 or anti-Cyt2 antibody diluted in a permeabilisation buffer (0.1% w/v saponin in PBS). T cells from each

condition were also incubated with a mouse IgG1 (Invitrogen. Carlsbad, CA, USA) antibody to measure background fluorescence. After incubation, the cells were washed 3 times with FACS buffer and incubated for 20 mins, in the dark, with the fluorescent rat anti-mouse IgG-FITC secondary (BD Bioscience, USA). Finally, cells were washed 3 times with FACS buffer and resuspended in 200 µl FACS buffer. T cells were then analysed for their cell intracellular expression of Cyt1 and Cyt2 with the BD Biosciences FACScan flow cytometer using Cell Quest software. $CD4^+$ cells were gated based on the forward and side scatter to exclude debris. The data was exported and then analysed using FlowJo 8.8.4 software (TreeStar USA).

### 2.7.3 Detection of phosphorylated LAT

In order to determine the expression of phosphorylated linker of activated T cells (p-LAT), T cells were activated with either αCD3 (50 µg/ml)/CD28 (50 µg/ml) in the presence or absence of αCD19 (50 µg/ml) in addition to an anti-mouse IgG crosslinker (Jackson Immunoresearch, USA) for 5 mins. The cells were immediately fixed (Cytofix buffer, BD Bioscience, USA) and permeabilised (Phosflow Perm III Buffer, BD Bioscience, USA). The cells were stained for p-LAT-PE (BD Bioscience, USA) for 20 mins in the dark and washed twice with FACS buffer. Finally, the cells were resuspended in 200 µl FACS buffer. T cells were then analysed for their cell expression of p-LAT with the BD Biosciences FACalibur flow cytometer. $CD4^+$ cells were gated based on the forward and side scatter to exclude debris. The data was exported and then analysed using FlowJo 8.8.4 software (TreeStar USA).

# 2.8 Cytokine detection

IL-10 and IFNγ secretion was determined by either enzyme-linked immunosorbent assay (ELISA) or a secretion assay depending on the experimental requirements.

## 2.8.1 ELISA

ELISA was used to determine IL-10 and IFNγ secretion from the supernatants of T cells that had been activated for 5 days (*section 2.4*). T cells suspensions were collected from the tissue culture plate and centrifuged at 6000 x g for 20 seconds. The supernatant was carefully removed from the eppendorf and transferred to a new tube, without disturbing the cell pellet. These supernatants were frozen at -20 $^{o}$C or used immediately for ELISA. IL-10 and IFNγ secretion was determined using an ELISA specific for human IL-10 (BD Pharmingen, San Jose, USA) and IFNγ (Pierce Biotechnology, Rockford, USA). Each well of high binding 96 well plates (Nunc, Rochester, NY) was coated with either 50 µl of IL-10 (1 in 500) or IFNγ (1 in 1000) diluted in a plate coating solution (0.1M $NaHCO_3$ in $ddH_2O$) and incubated for 2 hrs at room temperature. Each plate was washed 3 times with TBST (PBS with 0.05% v/v Tween 20 (Bio-Rad, Hercules, USA)). Then, each well was blocked for 2 hrs at room temperature with 200 µl of 1% w/v bovine serum albumin (BSA)(Roche Diagnostics Ltd, UK) in PBS. The blocking solution was discarded and the supernatants were loaded onto the plate. Samples were added in duplicate with the appropriate serial dilutions. Recombinant human IL-10 (BD Pharmingen, San Jose, USA) and human IFNγ (Pierce Biotechnology, Rockford, IL) were used as standards. After adding the samples and standards to the plates, they were incubated for 2 hrs at room temperature and then washed three times with TBST. Each plate was coated with 50 µl/well of either a biotinylated anti-human IL-10 (1 in 2000 in 1% w/v BSA-PBS) or biotinylated anti-human IFNγ (1 in 1000 in 1 % w/v BSA-PBS) and incubated for 1 hr at room temperature. Each plate was washed three times with TBST and coated with 50 µl per well of a streptavidin-horseradish peroxidase (HRP) (R&D

Systems, Minneapolis, USA) conjugate dilution (1 in 10,000 in 1 % w/v BSA-PBS). The plates were incubated for 1 hr at room temperature and washed three times with TBST. After the final wash, 50 µl per well of a tetramethylbenzidine (TMB) substrate kit (Invitrogen, Carlsbad, Ca, USA) was used as a substrate for HRP. The reaction was stopped with 50 µl per well of stop solution (1M $H_3PO_4$ in $ddH_2O$). Each plate was read at 450 nm using a BioTek microplate reader (Winooski, VT, USA). Results were exported to Microsoft Excel for analysis and Graphpad software was used for figure preparation.

### 2.8.2 Secretion assays

In order to assess the percentage of cells secreting IL-10 or IFNγ an IL-10-PE and IFNγ-APC secretion assay (Miltenyi Biotec, Auburn, CA, USA) were used. T cells were activated as described in *section 2.4*. After three days activation ~ 2 x $10^5$ T cells from each activation condition were collected in a 1.5 ml eppendorf and centrifuged for 5 min at 900 x g. The supernatants were discarded and the cells were washed with 1 ml of cold wash buffer (0.5 % w/v BSA and 2 mM EDTA in PBS). The cells were resuspended in 80 µl of cold 10 % RPMI 1640 culture medium (*see section 2.4*) and 3 µl of the αIL-10 and αIFNγ catch reagents (IL-10 or IFNγ specific monoclonal antibodies conjugated to a CD45 specific monoclonal antibody) were added to each tube. The eppendorfs were then incubated for 5 mins on ice before adding 1 ml of pre-warmed (37 $^oC$) 10% RPMI 1640 culture medium to each tube. To allow cytokine secretion to occur, the samples were incubated at 37 $^oC$ under slow continuous rotation for 45 mins. Then, the cells were washed with 0.5 ml of cold buffer and resuspended in 160 µl of cold wash buffer. Half of the cell suspension was transferred to a fresh tube labeled 'negative control'. To the remaining half of the cell sample 3 µl of the IL-10 (PE) and IFNγ (APC) detection antibody was added, mixed well and incubated on ice for 10 mins. Following this incubation period, each cell sample was washed once with 1 ml of cold buffer, resuspended in 160 µl of FACS buffer (*see section 2.7.1*) and IL-10 and IFNγ secretion was analysed on the BD

Biosciences FACSCalibur flow cytometer using Cell Quest software. The data was exported and analysed using FlowJo 8.8.4 software (TreeStar USA).

## 2.9 Proliferation assays

### 2.9.1 Thymidine incorporation

Freshly isolated T cells were plated at a concentration 5 x $10^4$ in a 96 well culture plate, pre-coated with the stimulating antibodies. Each activating condition was carried out in duplicate. T cells were activated for 72 hrs, before adding 1 μCi of [$^3$H]thymidine (Amersham) diluted in 20 μl of 10% RPMI 1640. After 24 hrs, proliferation was determined using a Liquid Scintillation Counter (Wallac, Boston, USA).

### 2.9.2 CFSE incorporation

To assess proliferation using carboxyfluorescein diacetate succinimidyl ester (CFSE) (eBioscience, San Diego, USA), freshly isolated $CD4^+$ T cell (2 x $10^6$) were span down in a 1.5 ml eppendorf and washed 3 times with 500 μl of ice cold PBS. Cells were incubated with CFSE at 37 $^o$C for 10 mins on a rotator in the absence of light. Ice cold 10% RPMI was added to cells and the samples were left for 4 mins on ice. Finally the cells were washed 3 times with cold medium and stimulated as previously described. After 4 days activation, the CFSE-labelled cells were harvested and proliferation levels were assessed by flow cytometry using the BD Biosciences FACSCalibur flow cytometer. The data was exported and then analysed using FlowJo 8.8.4 software (TreeStar USA).

## 2.10 Immunoprecipitation

In order to immunoprecipitate CD46 from supernatants or cell lysates, all reagents were kept on ice at all times and incubation steps were preformed at 4 $^o$C. When CD46 was immunoprecipitated from cell samples, 300 μl of radio

immunoprecipitation assay buffer **(**RIPA) lysis buffer (NP40 1% v/v, NaCL 150 mM, Tris Ph 7.6 50 mM, EDTA 5 mM, PMSF 1 mM, NaF 10 mM, NagVO4 25 mM, protease inhibitor cocktail 1 % v/v, SDS 0.08% w/v, DOC 0.5% w/v) was added to each sample. Then each sample was vortexed and incubated with the RIPA buffer for 10 mins. The lysates were centrifuged for 10 mins at 9200 x g and the supernatants were transferred to fresh tubes for immunoprecipitation.

Both cell supernatants and cell lysate samples underwent a pre-clear step using the protein G covalently bound to sepharose beads (GE Healthcare, UK). Protein G (20 µl diluted 1:1 with lysis buffer) was added to each sample and left to incubate for 45 mins on an orbital rotator. The samples were then centrifuged for 1 min at 4000 x g. The supernatants were carefully removed from the eppendorfs, taking care not to touch the protein G beads at the bottom of the tube, and transferred to fresh eppendorfs. Mouse αCD46 (1 µg in 50 µl RIPA buffer per sample, clone MCI20.6) was added to each tube and incubated for 2 hrs on an orbital rotator. Following incubation, 20 µl of protein G beads was added to each sample and incubated on the shaker for 1 hr. The samples were centrifuged at 4000 x g for 1 min and the supernatant was carefully removed, again taking care not to disturb the protein G beads, which were now bound to the CD46 antibodies. The protein G beads were washed twice with lysis buffer. After the final wash the supernatant was removed and 10 µl of 3x reducing sample buffer (glycerol 10% v/v, Tris pH 6.8 0.1M, bromophenol blue 0.02% w/v, 15% v/v β-mercaptoethanol) was added to each eppendorf. The samples were boiled at 96 $^{o}$ C for 10 mins. After boiling the samples were either frozen at -20 $^{o}$C or analysed directly by SDS-PAGE.

## 2.11 CD46 Deglycosylation

T cells were left unstimulated or stimulated in the presence of αCD3 (5 x $10^6$ per condition) as described in *section 2.4*. After 5 days activation, T cells were harvested and collected in 1.5 ml eppendorf tubes. The samples were washed

twice in PBS and CD46 was immunoprecipitated as described in *section 2.10*. However, instead of adding the sample buffer in the final step, the protein G pellets were washed once with deionised $dH_2O$, and were resuspended in 60 µl of deionised $dH_2O$. Both N- and O- glycans were removed from CD46 using the GlycoPro™ Enzymatic Deglycosylation Kit for N-Linked & Simple O-Linked Glycans (PROzyme, CA ,USA). Complex core 2 O-glycans were removed using the PRO-LINK Extender™ (PROzyme, CA, USA). Deglycosylation was carried out according to the manufacturers guidelines and all reagents used were supplied in the kits. In brief, 20 µl of reaction solution and 5 µl of the denaturation solution was added to each eppendorf. The samples were heated at 100 $^{o}$C for 5 mins. Once the eppendorfs had returned to room temperature 5 µl of detergent solution was added and mixed by flicking each tube. This suspension was split into two fresh eppendorfs tubes, one labelled "untreated" and the other labelled "treated". Then 1 µl of each of the deglycosylating enzymes (N-glycanase (PNGase F), O-glycanase (endo-α-N-acetylgalactosaminidase), sialidase A, β(1-4)-Galactosidase and β-N-Acetylglucosaminidase) was added to the 'treated' eppendorf, nothing was added to the 'untreated' samples. Both 'untreated' and 'treated' tubes were incubated for 24 hrs on an orbital rotator at 37 $^{o}$C. Finally, 15 µl of 3x sample buffer was added to each sample and heated at 96 $^{o}$C for 10 mins. The $M_w$ of CD46 samples was then analysed by SDS-PAGE or the samples were frozen at – 20 $^{o}$C for analysis at a later date.

## 2.12 SDS-PAGE and Western blot

In order assess CD46 expression and $M_w$, protein samples were first separated on an 8% acrylamide (Fisher Scientific, Loughborough, UK) gel by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PDVF, Millipore, Fisher Scientific, UK) membrane for CD46 protein detection.

### 2.12.1 SDS-page

The stacking and 8% acrylamide resolving gel were prepared using Bio-Rad equipment. The gels were placed in the electrophoresis tanks with running buffer (glycine 192 mM, Tris base 25 mM, SDS 0.1% in $ddH_2O$). Before loading samples onto the gel, the total cell lysates (cell pellets lysed in RIPA buffer) or CD46 immunoprecipitates were denatured using a 3x reducing sample buffer (*described in section 2.10*). The samples, a protein ladder (7-175 kDa, New England Biolabs, USA) and a total cell lysate (TCL) control (for CD46 immunoprecipitates) were reheated at 96 $^{o}$ C for 10 mins immediately prior to loading. After loading the samples, the gels were run at 150 V for ~10-15 mins and then at 200 V for 45 mins using a Bio-Rad powerpack. Following electrophoresis, the stacking gel was gently removed and the gels were washed twice with deionised water.

### 2.12.2 Western Blot

After SDS-PAGE was preformed, the separated proteins within the acrylamide gels were transferred onto PDVF paper using Bio-Rad transfer equipment. One PDVF membrane per gel was wet with 100 % ethanol and pre-soaked for ~20 mins in transfer buffer (Tris 20 mM, glycine 150 mM, ethanol 20% in $ddH_2O$). The gel and the PDVF membrane were tightly packed into transfer cassettes within a sandwich of Whatman chromatography paper and sponges. Each layer of the sandwich was pre-wet in transfer buffer and was placed on top of the previous layer in the absence of air bubbles. The cassette was closed and placed into the transfer tank with the black side of the cassette facing the black side of the electrode plate. The tank was filled to the top with transfer buffer and the lid secured ensuring that the colour-coded cables are matched with the electrode cards of the same colour. The transfer tanks were run at 100 V for 1 hr. Following the transfer of the proteins onto the membrane, the membrane was carefully removed and incubated for 1 hr on an orbital shaker in blocking buffer (5% w/v nonfat dry milk (Carnation, Nestle) in Tris-buffered saline and Tween 20 (TBST) (50 mM Tris base, NaCl 150 mM and 0.05% v/v Tween 20)). After blocking, the

membranes were incubated with a polyclonal rabbit αCD46 (2 µl in 10 mls of blocking buffer, Santa Cruz, USA) for 2 hrs at room temperature or overnight at 4 $^{o}$C. The membranes were washed five times with TBST over a period of 1 hr on an orbital shaker. Antibody bound proteins were then labelled with HRP-linked anti-rabbit IgG (1 µl in 10 mls of TBST, Promega, Madison, USA) for 1 hr. Following this incubation, the membranes were again washed 5 times over a period of 1 hr. Finally, the proteins stained with HRP were detected using the Lumiglo® chemiluminescent substrate (Cell Signaling, Beverly, USA). The emitted light was captured using X-ray film (Amersham Hyperfilm ECL, GE Healthcare, UK). The X-ray film was then scanned into a digital format using the KODAK ESP scanner and figures were prepared using Photoshop CS4. As a loading control, the membranes were also reblotted with a mouse anti-GAPDH monoclonal antibody (1 µl in 10 mls of blocking buffer, clone 6C5, Immunochemistry, Bloomington, USA) and a HRP-linked αmouse IgG secondary (1 µl in 10 mls of TBST, Promega, Madison, USA).

## 2.13 Confocal microscopy

The localisation of the ectodomain of CD46 and the intracellular tails, Cyt1 and Cyt2 were examined using confocal microscopy. Chamber slides (16 well, Nunc, VWR International Ltd, UK) were pre-coated with poly-D-lysine (PDL) ($M_w$ 70,000-150,000, 2µg/ml) and dried thoroughly before coating the well with stimulating antibodies. The wells were pre-coated as described early with either mouse IgG (15 µg/ml) or αCD3 (5 µg/ml) and αCD46 (10 µg/ml). Freshly isolated $CD4^+$ T cells were cultured for 2 or 4 days at a concentration of 2 x $10^5$ per well. At the defined activation timepoint, T cells were gently washed with pre-warmed PBS before fixating the cells for 10 mins with 4% paraformaldehyde (PFA). The PFA was gently removed and the cells were washed twice with PBS. Following this step, PFA aldehyde groups were quenched with $NH_4Cl$ (50 mM in PBS) for 15 mins. $NH_4Cl$ was removed and the cells were washed twice with PBS before permeabilisation with 0.5% v/v Triton X-100. Then, non-specific binding sites were blocked by incubating the cells for 2 hrs, at room temperature,

in the presence of a blocking buffer (20% v/v goat serum in PBS, Cambridge Bioscience, UK). The blocking buffer was removed and the T cells were incubated with mouse αCD46 (clone MCI20.6), αCyt1 or αCyt2 monoclonal antibodies overnight on an orbital shaker at 4 $^{o}$C. After extensive washing, cells were fluorescently labelled with the Fab (ab')2 fragment of goat anti-mouse IgG Alexa Fluor-488 (Invitrogen, Carlsbad, CA, USA) for 1 hr at room temperature. After washing, the cells were counterstained with DAPI nuclear stain (2 μg/ml) and mounted using ProLong Gold (Invitrogen, Carlsbad, CA, USA). Images were acquired using the Leica SP5 and imported to ImageJ 1.42q for analysis.

## 2.14 Statistical analysis

Statistical analysis was preformed using Graphpad Prism software. All data comparisons within the same donor group were analysed using a non-parametric paired Friedman test and a Bonferroni-corrected Wilcoxon test. All data analysis between healthy control and patient groups used the non-parametric unpaired Kruskal-Wallis test and Bonferroni-corrected Mann-Whitney Test. All p-values are two-tails and with a 95% confidence interval. Bar charts are plotted as the mean and ± SEM (Standard Error Mean). Box and whisker plots are represented with Tukey whiskers.

# Chapter 3 : Expression and Regulation of CD46 during T cell activation

## 3.1 Introduction

Over 20 years ago, CD46 was identified as a member of the complement regulatory receptors (Cole et al., 1985, Seya et al., 1986, Lublin et al., 1988). Later, CD46 was identified as a T cell costimulatory receptor (Astier et al., 2000) that can induce a regulatory (Kemper et al., 2003, Cardone et al., 2010) or pro-inflammatory response (Sanchez et al., 2004, Cardone et al., 2010). The importance of CD46 in T cell responses is underlined by its regulatory defects in multiple sclerosis (Astier et al., 2006, Martinez-Forero et al., 2008, Ma et al., 2009), asthma (Xu et al., 2010, Tsai et al., 2012) and rheumatoid patients (Cardone et al., 2010). Moreover, the expression of CD46 is reduced in T cells from asthma patients (Tsai et al., 2012), SLE patients (Alegretti et al., 2012) and increased in cancer cells indicating a relationship between dysregulated expression and function. Two factors that are fundamental to receptor activity are the presence of its cognate ligand and its own expression levels. Therefore, determining CD46's pattern of expression during activation may shed light on the role of CD46 during T cell activation.

CD46's effect on T cell phenotype is known to be plastic depending on the timing of its ligation (Oliaro et al., 2006) and soluble factors present in the *milieu* including IL-2 (Cardone et al., 2010), PGE-2 (Kickler et al., 2012) and vitamin D (*manuscript in press*). The expression levels of CD46 isoforms Cyt1 and Cyt2 also appear to play an important role in T cell responses (Marie et al., 2002, Cardone et al., 2010, Ni Choileain et al., 2011). Of note, in a transgenic mouse model, expression of the Cyt1 isoform in a contact hypersensitivity suppressed the inflammatory response, while Cyt2 promoted inflammation (Marie et al.,

2002). Furthermore, increased expression of Cyt2 mRNA has been identified in multiple sclerosis (Astier et al., 2006) and asthma patients (Tsai et al., 2012). The contrasting roles of CD46 cytoplasmic isoforms on inflammation is becoming increasingly relevant as our understanding of T cell biology is changing to incorporate the magnitude of T cell plasticity (reviewed in (Zhou et al., 2009, Bluestone et al., 2009, Murphy and Stockinger, 2010, Nakayamada et al., 2012)). Therefore the expression levels of Cyt1 and Cyt2 could impact upon T cell phenotype and may have important consequences for the regulation of immune responses.

Mechanisms that regulate CD46 expression have been previously reported. Specifically, downregulation of CD46 has been observed upon its ligation (Naniche et al., 1993, Gill et al., 2003, Elward et al., 2005, Hakulinen and Keski-Oja, 2006, Cole et al., 2006, Sakurai et al., 2007, Lovkvist et al., 2008, Mahtout et al., 2009, Basmarke-Wehelie et al., 2011). CD46 surface cleavage by MMP/ADAM(s) in epithelial, neuronal and cancer cells has been documented during cell death or pathogen infection (Van Den Berg et al., 2002, Hakulinen and Keski-Oja, 2006, Cole et al., 2006, Mahtout et al., 2009, Weyand et al., Basmarke-Wehelie et al., 2011). After commencing this project, presenilin-γ-secretase (PγS) cleavage of Cyt1 and Cyt2 intracellular tails was also shown to occur upon *Neisseria* infection of epithelial cells (Weyand et al.). However, prior to this investigation CD46 proteolysis had not been addressed in T cells (Ni Choileain et al., 2011). Downregulation of CD46 has also been attributed to internalisation (Naniche et al., 1993, Crimeen-Irwin et al., 2003) which often precludes degradation. Proteolytic cleavage and internalisation can play a role in terminating receptor responses but they can also initiate receptor signalling (Parks and Curtis, 2007, Fortini, 2009). Thus, investigations of these pathways in T cells will advance our understanding of CD46 function in regulating T cell responses.

Herein, the previously unknown patterns of CD46 expression and processing during human T cell activation are elucidated. Upon CD46 costimulation, CD46 expression levels are tightly regulated and undergo proteolytic cleavage within its extracellular and intracellular domains. Moreover, there is distinct expression levels of CD46 upon CD28 costimulation compared to CD46 costimulation. In addition, expression levels are inherently linked to TCR induced T cell activation and are suggestive of a key role for CD46 during T cell activation.

## 3.2 Aims

1. To determine the cell surface expression of CD46 upon human T cell activation
2. To determine the expression of the Cyt1 and Cyt2 isoforms upon human T cell activation.
3. To identify processes that regulate CD46 expression.

## 3.3 Approach

In order to understand the role of CD46 in regulating $CD4^+$ T cell activation, expression levels of CD46 were determined over a five-day period of activation. $CD4^+$ T cells were activated *in vitro* by different combinations of plate bound antibodies (*figure 3.1*). Surface expression levels were determined alongside alterations in expression of the isoforms Cyt1 and Cyt2 (*figure 3.1*). Of note, *in vitro* stimulation is limited in its ability to imitate temporal and spatial factors of *in vivo* T cell activation. As discussed in *Chapter 1*, antigen signal strength, cytokines present in the environmental *milieu* and the complex array of costimulating signals that a T cell receives from an APC also play an important role in determining the differentiation state of a T cell upon activation. Very little is known about CD46 costimulation *in vitro* or *in vivo* and the simplified approach utilized here to analyse CD46 expression levels is an important initial step in understanding the role of CD46 in more complex *in vivo* studies.

After analysis of CD46 expression levels during *in vitro* T cell activation, proteolytic cleavage of CD46 by MMPs and ADAMs was analysed using the broad-spectrum MMP/ADAM inhibitor, GM001 (*figure 3.1*). Culture supernatants were analysed for soluble CD46 (sCD46) fragments to confirm if any cleavage occurred. In order to examine if PγS cleavage of Cyt1 and Cyt2 also occurred, PγS cleavage was blocked using the inhibitor DAPT.

The role of internalisation and degradation in regulating CD46 expression was also investigated. Firstly, intracellular staining by flow cytometry was performed to determine if internalisation of CD46 had occurred (without degradation) upon T cell activation. Secondly, broad-spectrum inhibitors of both lysosomal and proteasome degradation were used to determine if CD46 expression could be regulated by degradation during T cell activation.

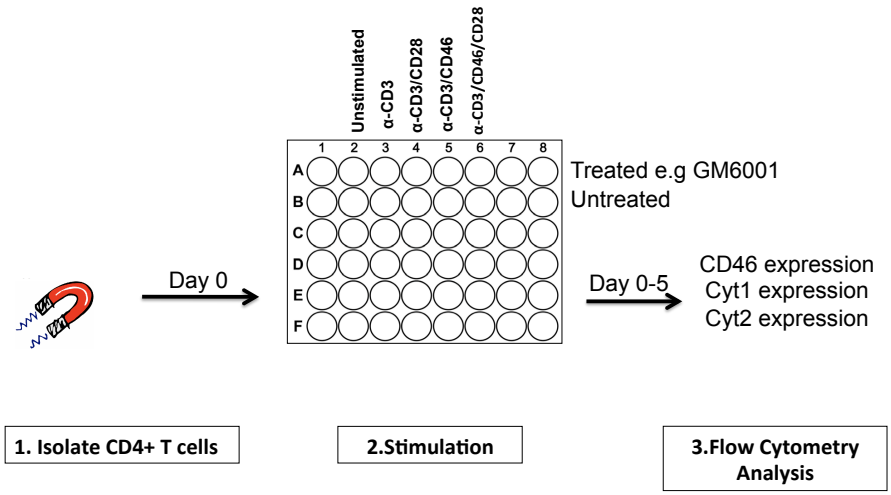


***Figure 3.1: Experimental setup for T cell stimulation and CD46 expression analysis.*** *T cells were left unstimulated (US) or activated in the presence of αCD3, αCD3/CD28, αCD3/CD46, and/or αCD3/CD28/CD46. In some experiments T cells were stimulated in the presence of broad-spectrum or specific inhibitors i.e. GM6001 (MMP/ADAM), DAPT (PγS), Chloroquine (lysosomal degradation) or MG132 (proteasome degradation). Expression of CD46 (extracellular and/or intracellular) and it's intracellular cytoplasmic tails (Cyt1 and Cyt2) was determined by flow cytometry at different time-points over a 5 day stimulation period.*

# 3.4 Results

## 3.4.1 CD46 is downregulated from the cell surface of T cells upon CD46 costimulation

To determine the cell surface expression levels of CD46 upon T cell activation, purified human $CD4^+$ T cells were left unstimulated (US) or activated with αCD3 or αCD3/CD46. Cell surface expression of CD46 was examined by flow cytometry after overnight stimulation. A striking downregulation of CD46 was observed upon costimulation compared to both unstimulated and CD3 stimulated T cells. A small but significant increase in CD46 expression was also observed upon CD3 stimulation (*figure 3.2A, 3.2B*). Surface expression was also determined using two different CD46 antibody clones MCI20.6 and E4.3. Downregulation of expression was observed with both clones, although with different kinetics (*figure 3.2C*). Therefore, CD46 cell surface expression is downregulated upon CD46 costimulation in human $CD4^+$ T cells.

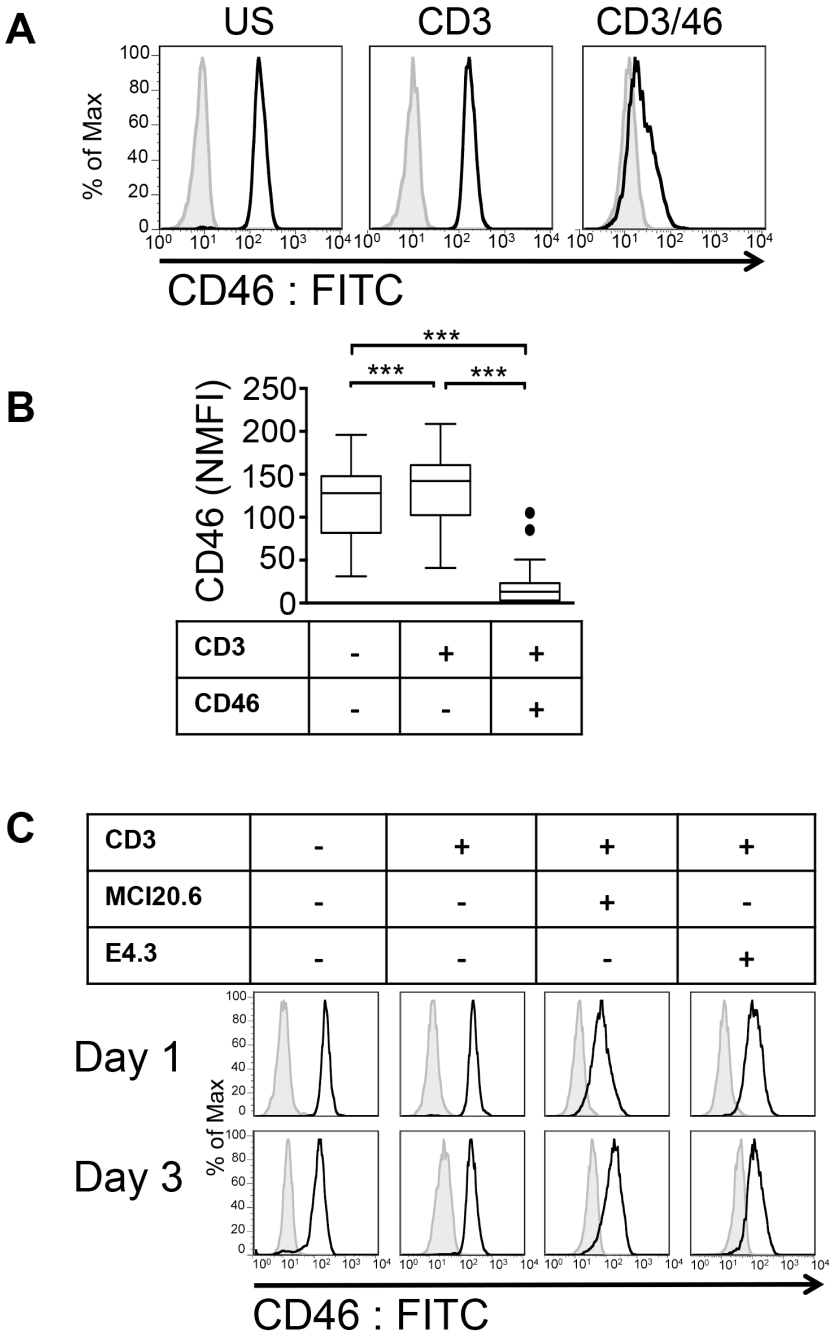


***Figure 3.2: CD46 is downregulated from the cell surface of T cells upon CD46 costimulation.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 (CD3) or αCD3/CD46 (CD3/46). Surface expression was determined by flow cytometry after 1 or 2 days stimulation.* ***(A)*** *Representative expression of cell surface CD46 following overnight simulation.* ***(B)*** *Mean CD46 expression after 1-2 days stimulation (n=25).* ***(C)*** *T cells were costimulated with either MC120.6 or E4.3 and surface CD46 expression was determined after 1 or 3 days stimulation (n=1). NMFI – normalised mean fluorescence intensity. A Friedman test and a Bonferroni-corrected Wilcoxon test was performed for statistical analysis ***p≤0.0003.*

### 3.4.2 CD46 downregulation is dose-dependent

There was a dose-dependent decrease in cell surface CD46 expression in the presence of increasing concentrations of αCD46 (2, 10, 20 μg/ml). The presence of αCD3 (5 μg/ml) remained constant. Of note, a non-specific IgG1 antibody was added to lower concentrations to ensure an equal volume of plate bound antibody (*figure 3.3*). In order to assess if decreasing surface expression of CD46 was due to cross-blocking by the stimulating antibodies, T cells were left unstimulated or activated overnight with either αCD3 alone or in the presence of αCD46 (10 μg/ml). As the activating antibodies were of mouse origin an anti-mouse-FITC was used to detect any binding of the stimulating antibodies to cells. No positive staining was observed (*figure 3.3B*) suggesting that the stimulating antibodies did not detach from the plate and bind to the cell surface of cells. Therefore, these data suggests that CD46 cell surface downregulation is dose dependent.

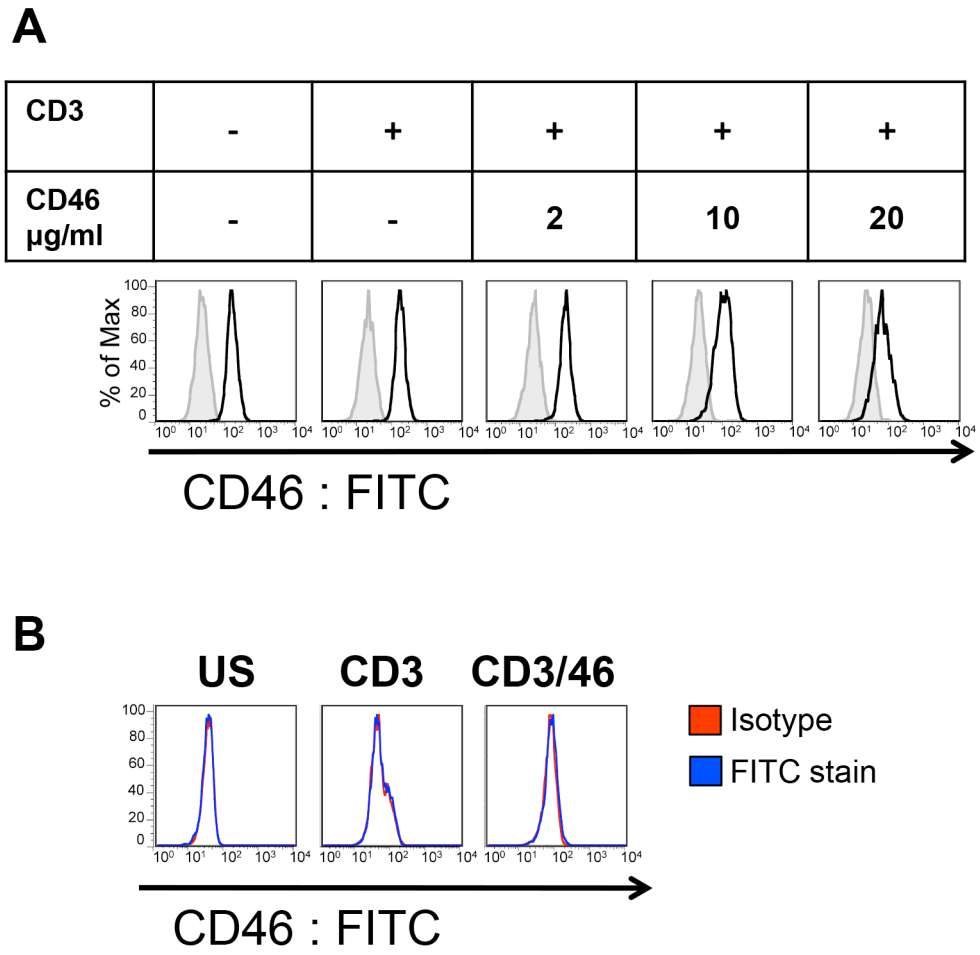


***Figure 3.3: CD46 downregulation is dose-dependent. (A)*** *T cells were left unstimulated or activated in the presence of αCD3 (CD3) alone or in the presence of an increasing dose of αCD46 (2, 10 or 20 µg/ml). Cell surface expression of CD46 was determined by flow cytometry after overnight stimulation, (n=1).* ***(B)*** *T cells were left unstimulated or activated in the presence of αCD3 (CD3) alone or in the presence of αCD46. After overnight stimulation cells were incubated with an anti-mouse-FITC antibody (n=1).*

### 3.4.3 CD46 downregulation is time-dependent

CD46 cell surface expression was then observed over several time-points: 1, 2, 3 hrs and each day for 5 days. Upon CD46 costimulation, CD46 downregulation was observed as early as 2 hrs after activation and was maintained over 5 days of stimulation (*figure 3.4*). Although CD46 remains downregulated compared to controls, expression levels increase slightly from days 2-4 (*figure 3.4B)*. Therefore CD46 downregulation is time-dependent.

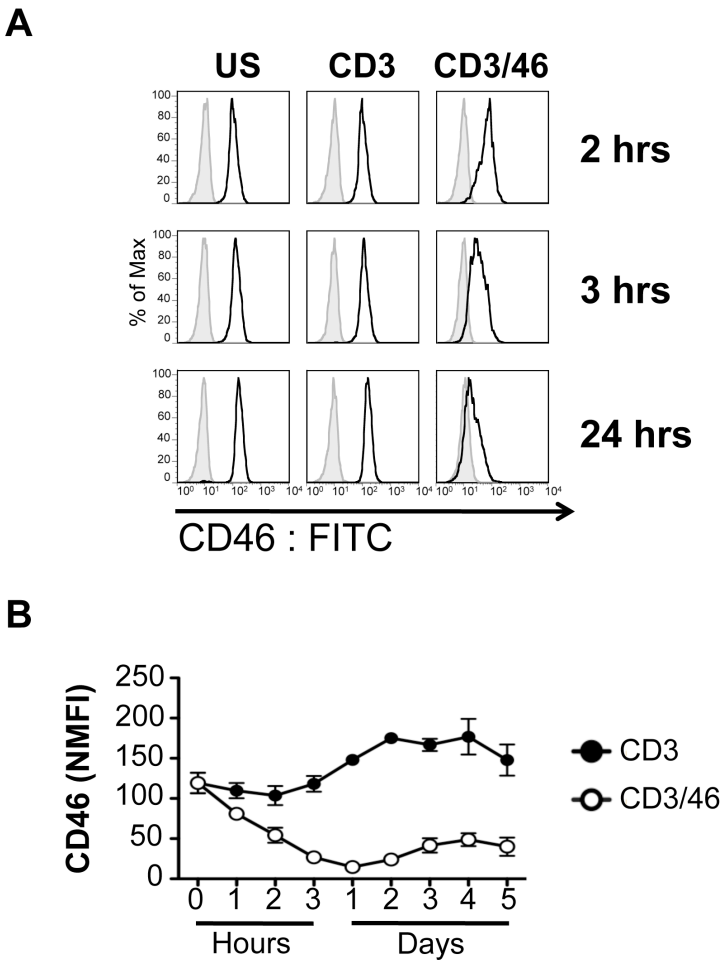


***Figure 3.4: CD46 downregulation is time-dependent.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 (CD3) or αCD3/CD46 (CD3/46). Surface expression was determined by flow cytometry over a period of 5 days.* ***(A)*** *Representative plot of CD46 downregulation at 2, 3 and 24 hrs (n=3).* ***(B)*** *Mean expression of cell surface CD46 expression upon CD3 or CD3/46 stimulation at different timepoints over a period of 5 days. (n=3).*

### 3.4.4 In the absence of CD46 ligation T cell activation induces an increase in surface CD46 expression

CD28 is one of the most dominant T cell costimulatory molecules and inhibition of its signalling pathway results in suppressed immune responses (Sperling and Bluestone, 1996). Therefore, surface expression levels of CD46 upon CD28 costimulation were also examined. Purified human $CD4^+$ T cells were left unstimulated or activated with αCD3, αCD3/CD28 or αCD3/CD46. CD46 surface expression was determined after 1-2 days activation (early activation) or 4-5 days activation (late activation). An increase in CD46 expression was observed upon CD28 costimulation at the early and late activation timepoints (*figure 3.5A and figure 3.5B*). In order to determine if a similar increase was observed with CD28's natural ligand, recombinant B7 was also used to stimulate T cells in the presence of CD3. In the presence of B7 (2.5 μg/ml) costimulation there was an increase in CD46 expression compared to CD3 stimulation alone (*figure 3.5C*). Moreover, overnight stimulation with PMA and ionomycin also induced an increase in CD46 expression. The activation marker, CD69, was also increased upon PMA/ionomycin treated confirming that the T cells were activated (*figure 3.5D*). Of note CD46 expression was also determined upon αCD3/CD28/CD46 (CD3/28/46) costimulation. Overall, these data indicate that T cell activation increases CD46 cell surface expression.

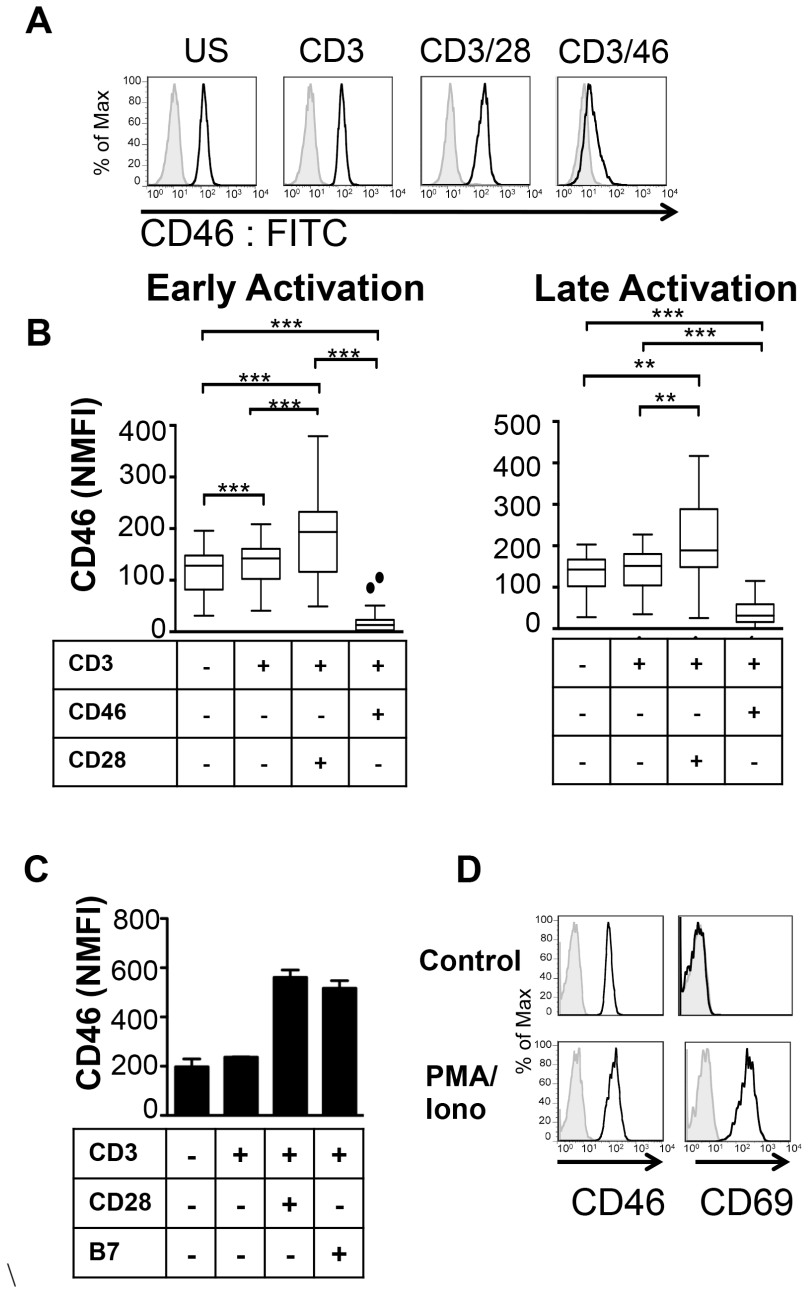


***Figure 3.5: T cell activation increases CD46 expression in the absence of CD46 ligation.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 (CD3) alone or in the presence αCD28 (CD3/28) or αCD46 (CD3/46). CD46 expression was determined by flow cytometry.* ***(A)*** *Representative CD46 expression after overnight stimulation.* ***(B)*** *Mean CD46 expression after 1-2 days stimulation (**Early Activation**) (n=25) and after 4-5 days stimulation **(Late activation**) (n=18)* ***(C)*** *T cells were left unstimulated or stimulated with αCD3 alone or in the presence of αCD28 mAb (CD28) or B7 for 3 days (n=2).* ***(D)*** *T cells were left unstimulated (control) or stimulated with PMA/ionomycin (n=2). NMFI –normalised mean fluorescence intensity. A Friedman test and a Bonferroni-corrected Wilcoxon test was used for statistical analysis ***p≤0.00025, **p≤0.0025.*

### 3.4.5 CD46 undergoes proteolytic cleavage by MMP/ADAM(s) upon CD46 costimulation

As CD46 was strongly downregulated upon CD46 costimulation it was next investigated how surface expression was downregulated. The mechanism for CD46 downregulation could have important consequences for its function. As CD46 was previously shown to undergo surface proteolysis in cancer cells (Hakulinen et al., 2004), dying cells (Elward et al., 2005, Hakulinen and Keski-Oja, 2006, Cole et al., 2006) and upon pathogen ligation (Van Den Berg et al., 2002, Mahtout et al., 2009, Weyand et al., Basmarke-Wehelie et al., 2011), we examined the possibility that CD46 also underwent proteolysis upon its ligation in T cells. T cells were left unstimulated or activated with αCD3, αCD3/CD46, or αCD3/CD28. T cells were cultured in the presence of a broad-spectrum MMP/ADAM(s) inhibitor, GM6001, or DMSO as a control. No effect of the inhibitor was observed in unstimulated, CD3 or CD28 costimulated cells. However, upon CD46 costimulation there was a significant increase in CD46 expression compared to DMSO treated cells (*figure 3.6A and figure 3.6B*). CD28/CD46 costimulation also induced the downregulation of CD46, which was also partially restored in the presence of GM6001 (*data not shown*). The presence of sCD46 was also detected in the supernatant of CD3/CD46 and CD3/CD28/CD46 costimulated T cells. Importantly, upon the addition of GM6001, there was a decrease in the level of sCD46 (*figure 3.6C- this experiment was kindly carried out by the BSc summer student Darragh Craughwell*). This suggests that upon CD46 costimulation CD46 is proteolytically cleaved by MMP/ADAM(s). Proteolytic cleavage of CD46 could have important functional consequences for cell signalling and also the environment *milieu* into which the sCD46 was released.

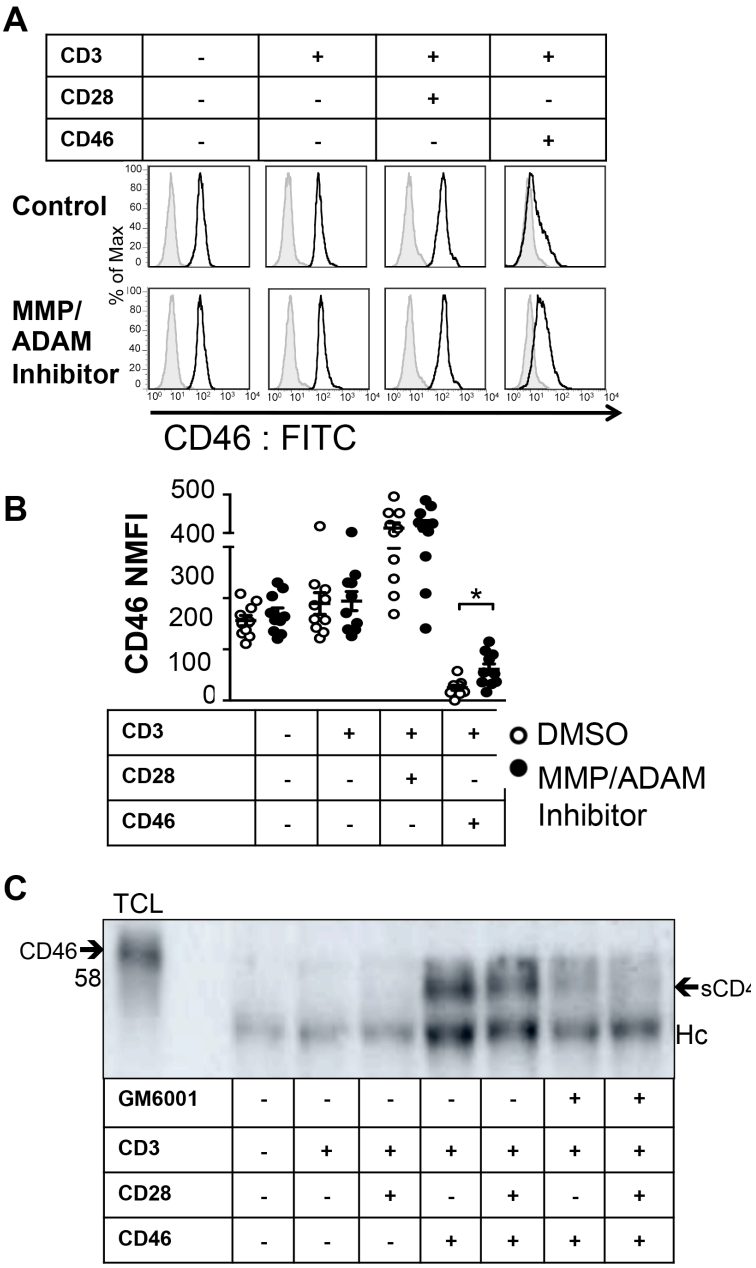


***Figure 3.6: CD46 undergoes proteolytic cleavage by MMP/ADAM(s) upon CD46 costimulation.*** *T cells were cultured in the presence of the MMP/ADAM inhibitor, GM6001, or DMSO as a control and left unstimulated or activated in the presence of αCD3 alone or in the presence of αCD46 and/or αCD28.* ***(A)*** *Representative CD46 expression determined by flow cytometry after overnight stimulation.* ***(B)*** *Mean CD46 expression (n=10), NMFI – normalised mean fluorescence intensity.* ***(C)*** *The level of sCD46 in the cell culture supernatants was determined by CD46 immunoprecipitation after the indicated stimulation. TCL = total cell lysate was loaded as a control for full length CD46 (Representative of 2 experiments). A Friedman test and a Bonferroni-corrected Wilcoxon test was used for statistical analysis *p≤0.01.*

### 3.4.6 CD46 is not retained intracellularly upon CD46 costimulation

CD46 costimulation induced a strong downregulation of cell surface CD46 expression that was partially the result of proteolysis. However, the MMP/ADAM(s) did not entirely restore CD46 cell surface expression. It was therefore hypothesised that CD46 was also internalised upon CD46 costimulation. Cross-linking of CD46 was previously shown to induce internalisation of CD46 in the Jurkat T cell leukaemia cell line (Crimeen-Irwin et al., 2003). In order to determine if CD46 was internalised upon CD46 costimulation, intracellular CD46 expression was determined after CD46 costimulation. T cells were left unstimulated or activated in the presence of αCD3 or αCD3/CD46 and intracellular CD46 expression was determined following overnight stimulation. Similar to the cell surface expression of CD46, intracellular staining also showed a decrease in CD46 expression upon CD46 costimulation compared to unstimulated cells (*figure 3.7A)*. However, the decrease observed was slightly less than that observed at the cell surface. This may be due to the presence of intracellular stores of CD46. Total CD46 expression was also determined by SDS-PAGE. Again a decrease in CD46 was observed (*figure 3.7B)*. Of note, as a result of O-glycosylation in CD46's STP region, CD46 can migrate as a single band or doublet, where the BC isoform migrates at ~66 kDa and the C isoform at ~56k kDa. In *figure 3.7B*, the blood donor expresses both isoforms and both have decreased expression upon CD46 costimulation. Finally, preliminary confocal studies were also carried out to determine CD46 expression and localisation upon activation. Of note, there were insufficient cell numbers to obtain a definitive result. Nonetheless, the preliminary results appeared to confirm that there was a decrease in CD46 expression upon CD46 costimulation (*figure 3.7C)*. Therefore, CD46 is not retained intracellularly upon its ligation. However, this does not exclude the possibility that CD46 is internalised and then rapidly degraded. As such, CD46 degradation was next assessed in CD46 costimulated T cells.

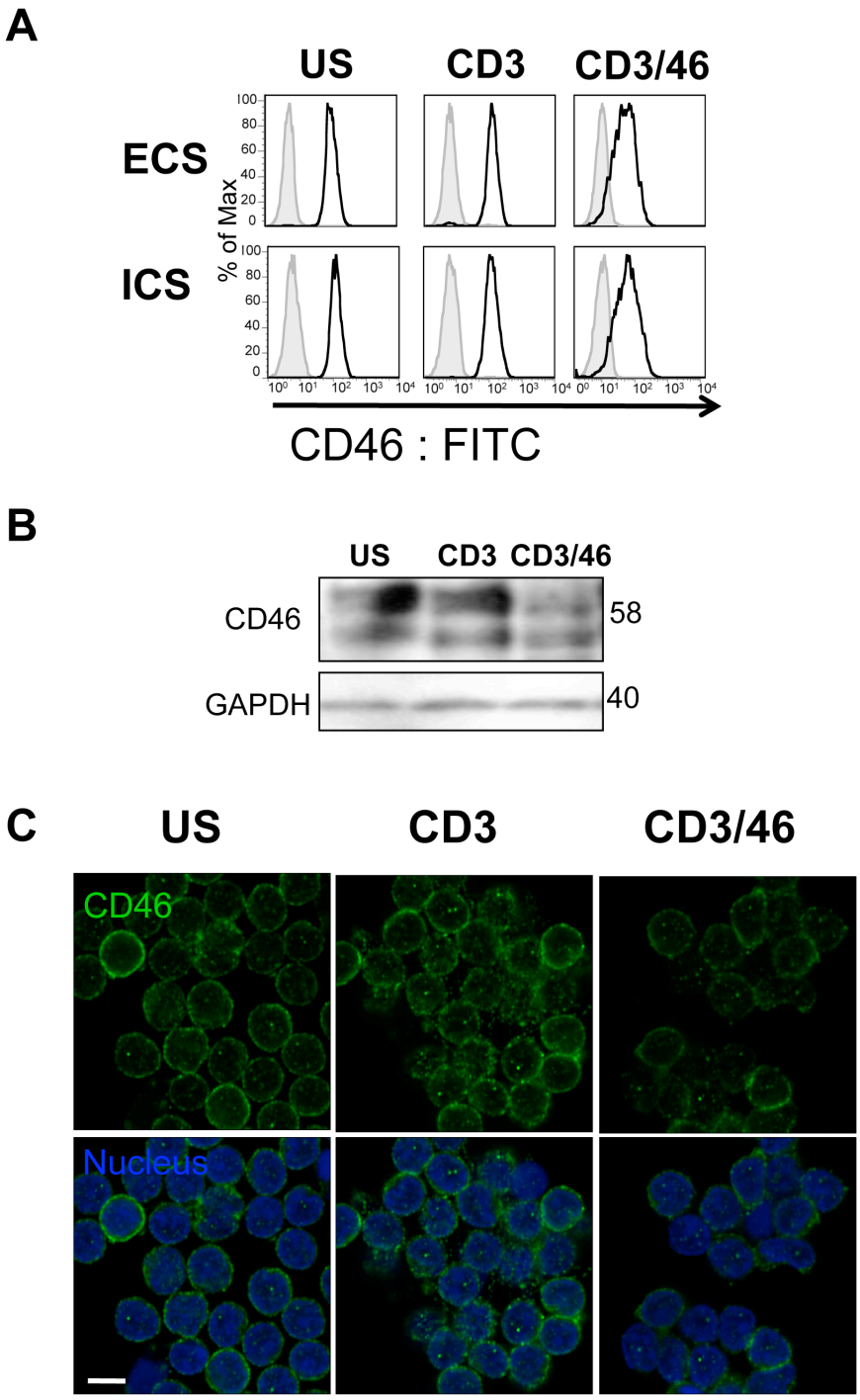


***Figure 3.7: CD46 is not retained intracellularly upon costimulation.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 (CD3) or αCD3/CD46 (CD3/46).* ***(A)*** *CD46 cell surface and intracellular expression after overnight stimulation as determined by flow cytometry (representative of 6 independent experiments)* ***(B)*** *CD46 expression after overnight stimulation as determined by SDS-PAGE (representative of 5 independent experiments).* ***(C)*** *CD46 surface expression was detected by confocal microscopy after over night stimulation.. (Top panel) CD46 staining, (Bottom panel) CD46 staining overlaid with DAPI, a nuclear counterstain. Scale bar 10 µm, (n=1).*

### 3.4.7 CD46 expression is increased upon inhibition of a chloroquine-sensitive pathway

Inhibition of CD46 downregulation with the MMP/ADAM inhibitor, GM6001, did not completely inhibit CD46 downregulation suggesting that decreases in CD46 surface expression may also be the result of internalisation. However, CD46 did not accumulate intracellularly, therefore the possibility that CD46 was degraded after internalisation was investigated. Indeed, it is known that cross-linking of receptors can target them for lysosomal degradation (Mellman, 1996) and CD46 contains the internalisation motif, YXXL motif, in the juxtamembrane of the Cyt1 and Cyt2 isoforms (Yant et al., 1997). Thus, both lysosome and proteasome degradation pathways were examined. Lysosomal degradation was firstly examined using the drug chloroquine. Chloroquine inhibits lysosomal degradation and prevents the fusion of endosomes, thereby inhibiting proper endosomal trafficking (Steinman et al., 1983). T cells were left unstimulated or activated with αCD3 or αCD3/CD46 in the presence or absence of increasing concentrations of chloroquine (2, 5, 10 μg/ml). At the highest dose, addition of chloroquine increased slightly the expression of surface CD46 upon costimulation (*figure 3.8A*). Upon CD46 costimulation intracellular expression of CD46 increased in the presence of both 5 and 10 μg/ml of chloroquine, with the strongest effect at the highest dose (*figure 3.8A*). At the highest dose there was also an increase in intracellular CD46 in all three activating conditions, with the strongest effect upon CD46 costimulation (*figure 3.8A and figure 3.8C*). Therefore, a chloroquine sensitive pathway negatively regulates CD46 expression and internalisation of CD46 via this pathway may have important consequences for T cell function.

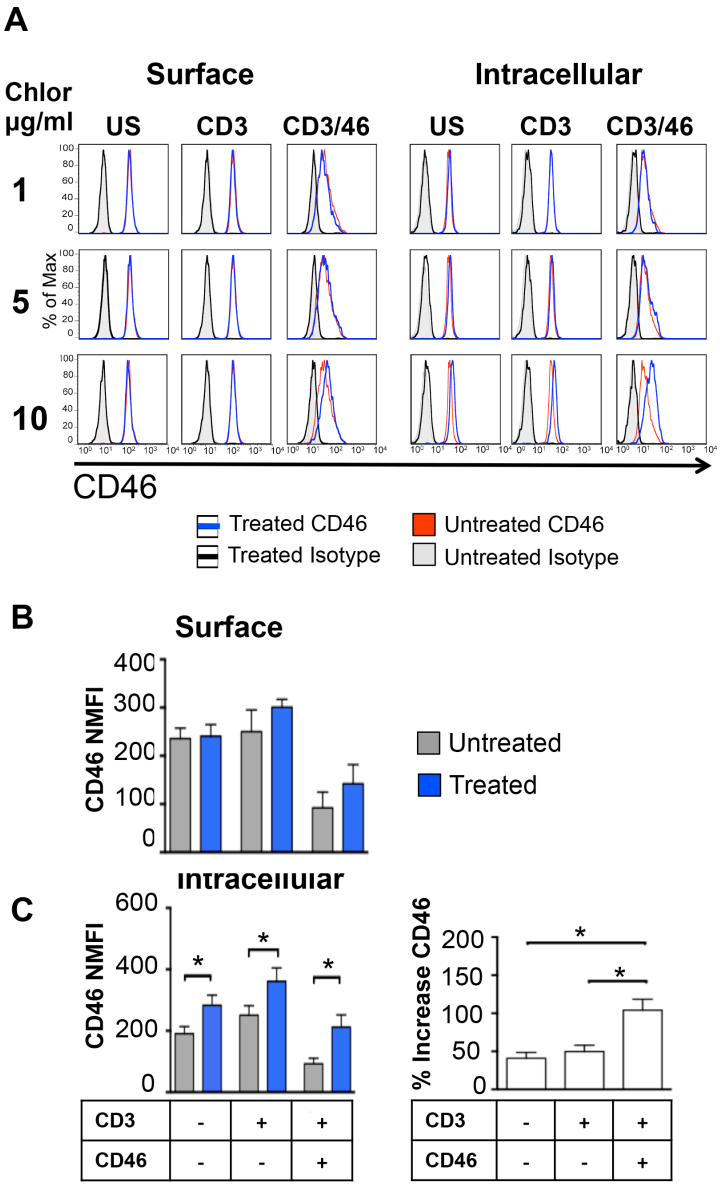


***Figure 3.8: CD46 expression is increased upon inhibition of a chloroquine-sensitive pathway.*** *T cells were either left unstimulated (US) or activated with αCD3 (CD3) or αCD3/CD46 (CD3/46). Cells were cultured for 2 days in the presence or absence of chloroquine and CD46 expression was determined by flow cytometry* ***(A)*** *Representative surface and intracellular CD46 expression after T cells were activated and cultured overnight with chloroquine (1, 5 or 10 µg/ml). CD46 expression of treated cells (blue) is overlaid with the untreated control (shaded grey).* ***(B)*** *Mean surface CD46 expression after cells were activated in the presence or absence of chloroquine (10 µg/ml) (n=3).* ***(C) (Left panel)*** *Mean intracellular CD46 expression after T cell activation in the presence or absence of chloroquine (10 µg/ml) (n=8).* ***(Right panel)*** *The percentage increase in intracellular CD46 expression in the presence of chloroquine compared to untreated controls (n=8). NMFI = normalised mean fluorescence intensity. Error bar = standard error mean. A Friedman test and a Bonferroni-corrected Wilcoxon test was performed for statistical analysis *p≤0.0167*

### 3.4.8 CD46 expression is decreased in the presence of the proteasome inhibitor, MG132

To examine if CD46 underwent proteosomal degradation, T cells were stimulated as above in the presence of increasing doses of the proteasome inhibitor MG132 (1, 5, 10 µM), or DMSO as a control. At the two higher doses MG132 dose-dependently decreased CD46 expression. The strongest effect was observed at the cell surface with smaller decreases observed intracellularly (*figure 3.9*). If indeed, CD46 were degraded by the proteasome, inhibition of its function would be expected to increase CD46 expression intracellularly. However, no increase in CD46 intracellular expression was observed and actually showed a slightly decreased expression of CD46 (*figure 3.9*). As CD46 expression was decreased in the presence of the inhibitor, it suggests that CD46 is not degraded by the proteasome after internalisation. However, further experiments need to be carried out to confirm this, as MG132 may indirectly affect CD46 expression levels.

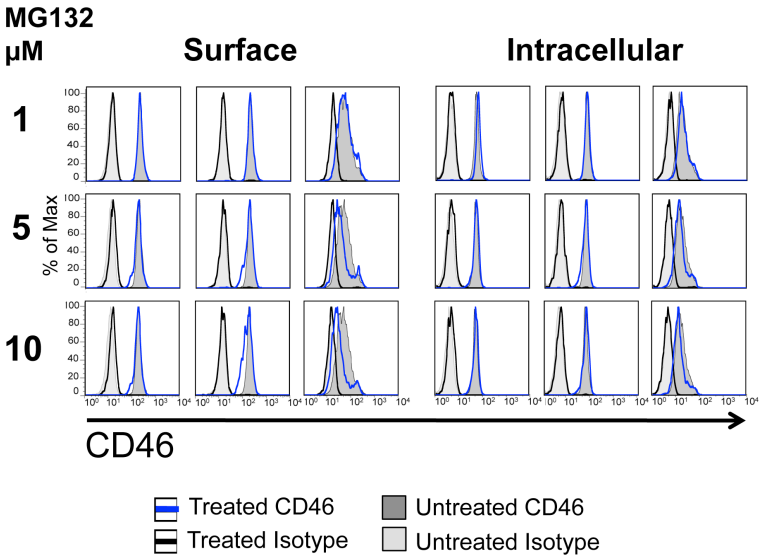


***Figure 3.9: CD46 expression is decreased in the presence of the proteasome inhibitor MG132.*** *T cells were cultured in the presence of the proteasome inhibitor, MG132, or DMSO as a control, left unstimulated (US) or activated in the presence of αCD3 (CD3) or αCD3/CD46 (CD3/46). CD46 expression was determined by flow cytometry after overnight stimulation* ***(A)*** *CD46 cell surface expression and* ***(B)*** *intracellular CD46 expression in T cells cultured with MG132 (1, 5 or 10 µM) or DMSO as a control. CD46 expression of treated cells (blue) is overlaid with the untreated control (shaded grey) after overnight activation, (n=1).*

### 3.4.9 Cyt1 and Cyt2 undergo time-dependent downregulation upon CD46 costimulation

In the transgenic mouse model that expressed either the Cyt1 or Cyt2 isoform, a contact sensitivity reaction was suppressed in the Cyt1 transgenic mouse and increased in the Cyt2 transgenic mouse (Marie et al., 2002). Due to the contrasting roles of the isoforms on inflammation in the murine model it was hypothesised that they would also play an important role in human T cell regulation. Therefore, the expression levels of Cyt1 and Cyt2 were observed over 5 days of T cell activation. T cells were left unstimulated or activated with αCD3 or αCD3/CD46. Expression levels of Cyt1 and Cyt2 were determined by flow cytometry using specific monoclonal antibodies designed by our collaborators Dr. Nathan Weyand and Prof. Maggie So (University of Arizona, Tucson). Expression levels were determined at an early activation timepoint (after 1-2 days stimulation) or at a late activation timepoint (after 4-5 days stimulation). Upon CD46 costimulation, there is a significant decrease in Cyt1 expression compared to CD3 stimulated cells at early but not at the late activation timepoint (*figure 3.10A and figure 3.10B*). However, Cyt2 expression had a contrasting expression pattern during T cell activation. At the early timepoint, CD46 costimulation increased Cyt2 expression, whereas during late activation Cyt2 was downregulated compared to controls (*figure 3.10C and figure 3.10D*). Upon the calculation of the Cyt2:Cyt1 ratio in CD46 activated cells during early activation there was an increase in Cyt2:Cyt1 compared to controls, whereas at the late timepoint the Cyt2:Cyt1 ratio this increase was absent (*figure 3.10E*). In summary, upon CD46 costimulation Cyt1 is downregulated during early activation whereas Cyt2 is upregulated. At the late timepoint, Cyt2 undergoes a timely downregulation. Taken together, these data indicate that Cyt1 and Cyt2 have unique expression levels, suggesting that they have distinct functions during T cell activation.

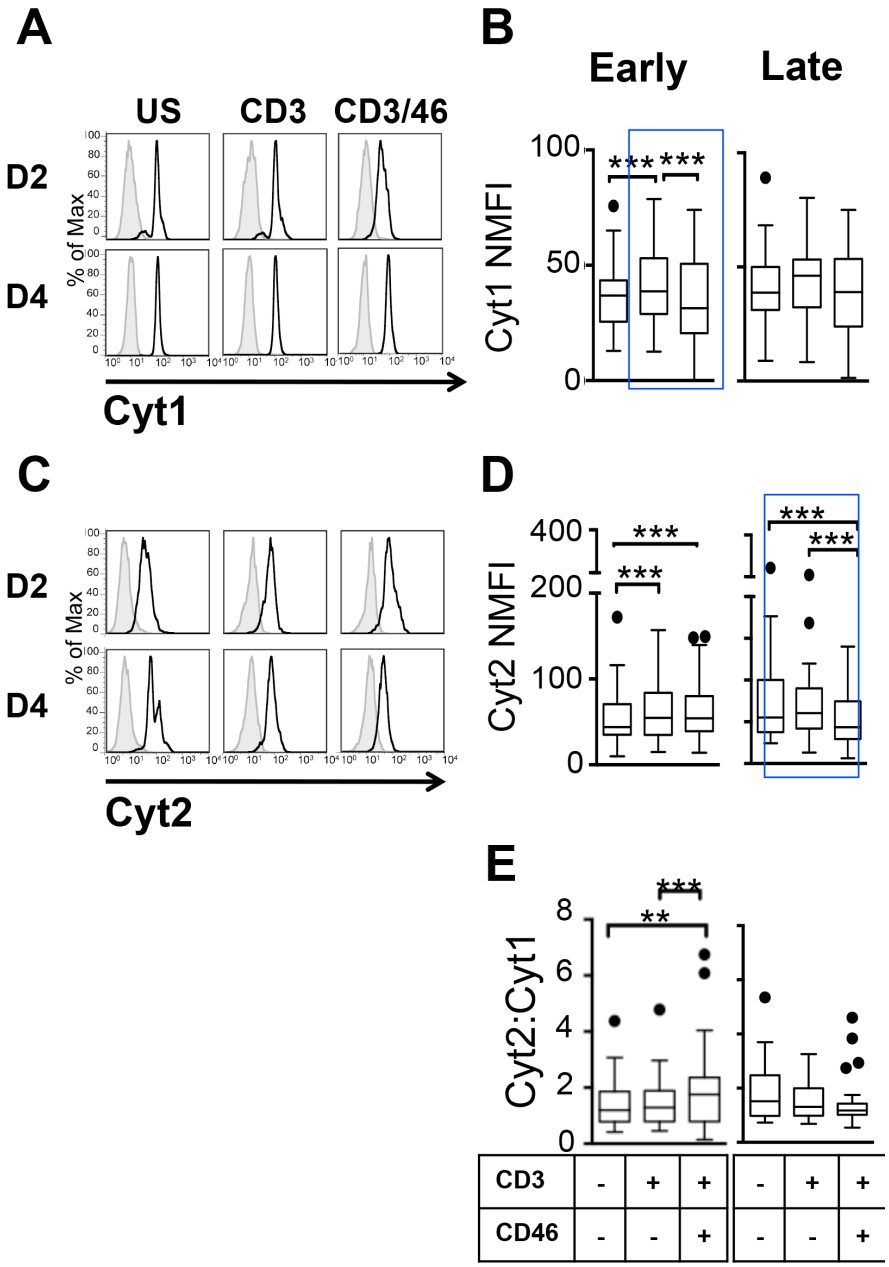


***Figure 3.10: Cyt1 and Cyt2 undergo time-dependent downregulation upon CD46 costimulation.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 (CD3) alone or in the presence of αCD46 (CD3/46). Cyt1 and Cyt2 expression were determined by flow cytometry after intracellular staining.* ***(A)*** *Representative Cyt1 expression after 2 and 4 days stimulation.* ***(B)*** *Mean Cyt1 expression at the early (n=40) and late activation (n=29) timepoint.* ***(C)*** *Representative Cyt2 expression after 2 and 4 days stimulation.* ***(D)*** *Mean Cyt2 expression at the early (n=40) and late activation (n=29) timepoint.* ***(E)*** *The ratio of Cyt2:Cyt1 expression. The timely downregulation of Cyt1 and Cyt2 is outlined by a blue box. NMFI – normalised mean fluorescence intensity. A Friedman test and a Bonferroni-corrected Wilcoxon test was performed for statistical analysis ***p≤0.0003,**p≤0.003,*p≤0.0167*

### 3.4.10 Cyt1 and Cyt2 expression is increased in the presence of the PγS inhibitor, DAPT

Both Cyt1 and Cyt2 underwent a time-dependent downregulation, suggesting an important functional significance. Thus, the downregulation of Cyt1 and Cyt2 was investigated further. After commencing this project, it was demonstrated by our collaborators that *Neisseria* ligation of CD46 in epithelial cells induced MMP cleavage, followed by cleavage of both Cyt1 and Cyt2 by PγS (Weyand et al.). As CD46 T cell costimulation resulted in MMP/ADAM(s) cleavage of CD46 (*figure 3.6*) it was hypothesised that the downregulation of Cyt1 and Cyt2 may be the result of PγS cleavage. Therefore, T cells were activated with αCD3/CD46 in the presence of the PγS inhibitor, DAPT, or DMSO as a control. Cyt1 and Cyt2 expression were determined at day 2 or day 4 by flow cytometry. Indeed, DAPT inhibited Cyt1 downregulation at day 2 and Cyt2 downregulation at day 4 (*figure 3.11*). PγS proteolysis of other transmembrane proteins, such as Notch, release intracellular domains (ICDs) with potent signalling capacities (Parks and Curtis, 2007). Therefore, the time-dependent proteolysis of Cyt1 and Cyt2 by PγS supports the hypothesis that Cyt1 and Cyt2 have distinct roles during T cell activation.

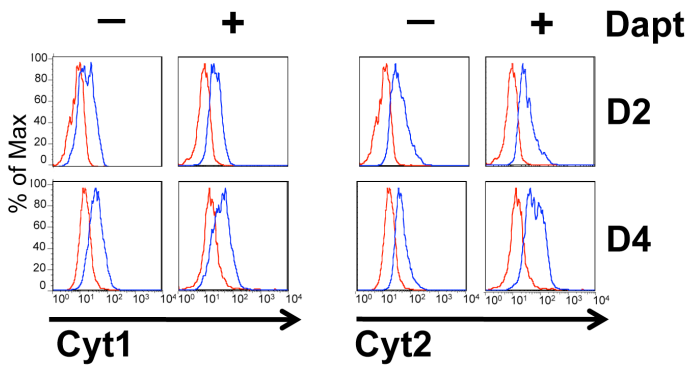


***Figure 3.11: Cyt1 and Cyt2 expression is increased in the presence of the PγS inhibitor DAPT.*** *T cells were activated with αCD3/CD46 and cultured in the presence of the PγS inhibitor, DAPT (10 μM) (+) or DMSO as a control (-). Expression of Cyt1 and Cyt2 was determined by flow cytometry after 2 or 4 days activation. Positive stain (blue histogram), Isotype control (red histogram) (Representative of four independent experiments).*

### 3.4.11 In the absence of CD46 ligation, Cyt1 and Cyt2 are differentially expressed during T cell activation

CD28 costimulation increased the expression of cell surface CD46, therefore the role of CD28 costimulation in regulating Cyt1 and Cyt2 expression was also defined. T cells were left unstimulated or activated with αCD3 or αCD3/CD28. Expression levels were determined at an early activation timepoint (after 1-2 days stimulation) or at a late activation timepoint (after 4-5 days stimulation). At both the early and late activation timepoints Cyt1 was increased in CD3/CD28 activated T cells. At the early timepoint Cyt1 was increased to a greater extent in the CD3/CD28 stimulated cells than CD3 stimulated cells (*figure 3.12A and figure 3.12B*). This increase in Cyt1 expression was in contrast to the observed results for CD46 costimulation where a downregulation of Cyt1 was observed at the early timepoint.

Upon CD28 costimulation, Cyt2 expression followed a similar pattern to Cyt1 expression during early activation and was increased in both CD3 and CD3/CD28 stimulated cells, again with the strongest increase upon CD28 costimulation. However at the late timepoint, Cyt2 showed a decreasing trend (*figure 3.12C and figure 3.12D*) similar to that observed upon CD46 costimulation. The ratio of Cyt2:Cyt1 was calculated at both timepoints. At the early timepoint upon CD28 costimulation, there was a decreasing trend in Cyt2:Cyt1 compared to CD3 stimulation and this decrease became significant at the later timepoint (*figure 3.12E*). As expected, costimulation in the presence of CD28 and CD46 ligation resulted in intermediate expression levels of Cyt1 and Cyt2 (*data not shown*). CD3 activation had similar patterns of Cyt1 and Cyt2 expression as CD28 costimulation, although these were less pronounced (*figure 3.12*). Therefore, even in the absence of CD46 ligation, T cell activation regulates both Cyt1 and Cyt2 expression during T cell activation and suggests an integral role for both Cyt1 and Cyt2 in T cell activation.

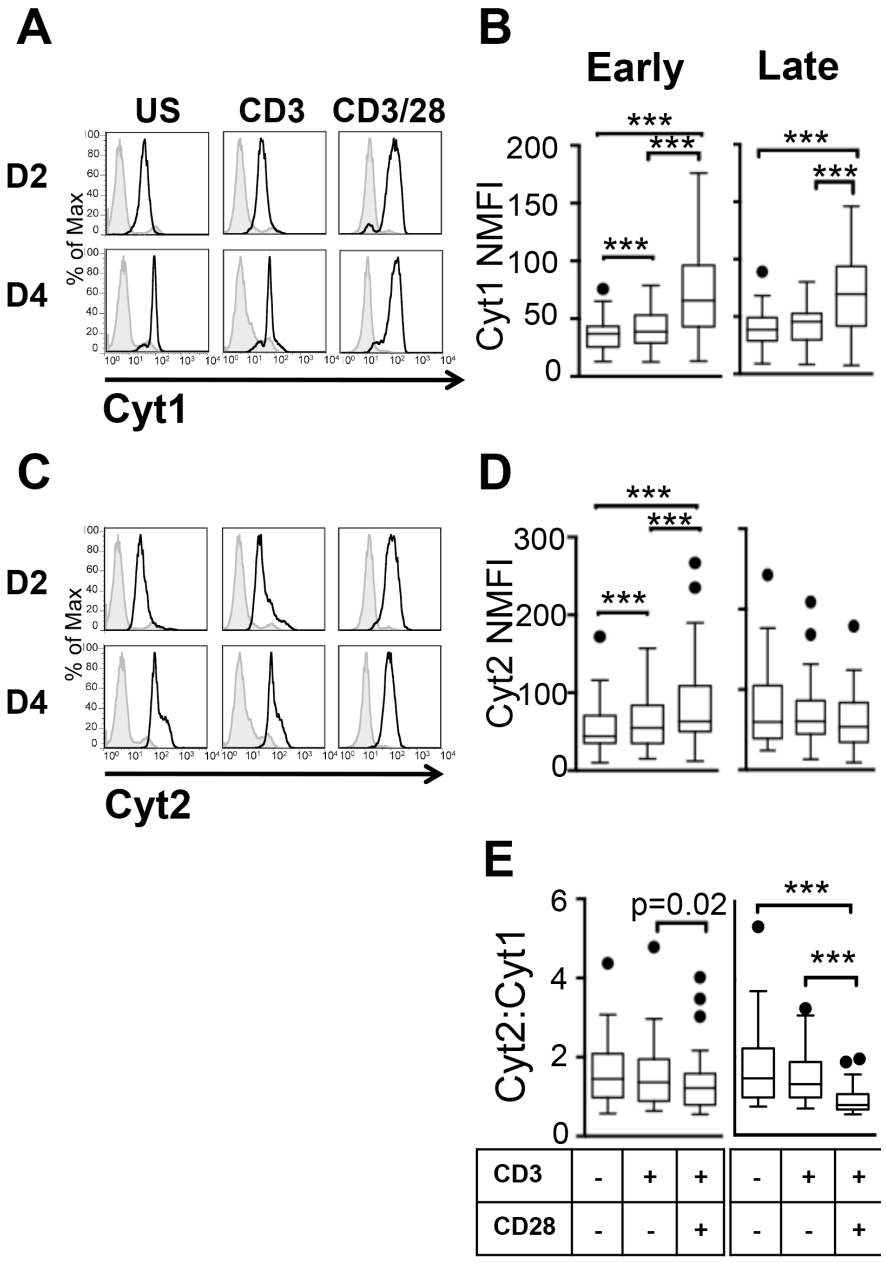


***Figure 3.12: In the absence of CD46 ligation, Cyt1 and Cyt2 are differentially regulated upon T cell activation.*** *T cells were left unstimulated (US) or activated in the presence of αCD3 alone (CD3) or αCD28 (CD3/28). Cyt1 and Cyt2 expression were determined by flow cytometry after intracellular staining.* ***(A)*** *Representative Cyt1 expression after 2 or 4 days stimulation* ***(B)*** *Mean Cyt1 expression during early activation (1-2 days stimulation) (n=40) or late activation (4-5 days stimulation) (n=29).* ***(C)*** *Representative Cyt2 expression after 2 or 4 days stimulation.* ***(D)*** *Mean Cyt2 expression during early activation (n=40) or late activation (n=29).* ***(E)*** *The ratio of Cyt2:Cyt1 expression during early and late activation. NMFI – normalised mean fluorescence intensity. A Friedman test and a Bonferroni-corrected Wilcoxon test was performed for statistical analysis ***p≤0.0003,**p≤0.003,*p≤0.0167*

### 3.4.12 IL-2 modulates CD46 expression

Finally, as IL-2 is added to all αCD3/CD46 cultures to induce a regulatory phenotype, the role of IL-2 on CD46 surface expression was determined. T cells were costimulated in the absence or presence of Il-2 (10 U/ml) for four days. CD46 expression was determined by flow cytometry at an early activation (1-2 days stimulation) or late activation (4-5 days stimulation) timepoint. There was a slight increase in CD46 surface expression upon the addition of IL-2 at the early timepoint. However, there was no significant difference during late activation (*figure 3.13A*).

As IL-2 is an important determinant for the CD46 induced Tr1-like phenotype, its role in regulating both Cyt1 and Cyt2 expression was also monitored. T cells were left unstimulated or activated with αCD3 or αCD3/CD46 with/without IL-2. Cyt1 and Cyt2 expression was determined by flow cytometry during early and late activation. At the early timepoint, there was an increase of Cyt1 in the presence of IL-2 (*figure 3.13B*). Cyt2 also showed a slight increase in the presence of IL-2 but the effect was not significant (*figure 3.13C*). At the later timepoint, there was no effect on either Cyt1 or Cyt2 (*figure 3.13B, 3.13C*). Therefore, IL-2 promotes increased Cyt1 expression and this reflects the increase in surface expression upon CD46 costimulation observed at day 2.

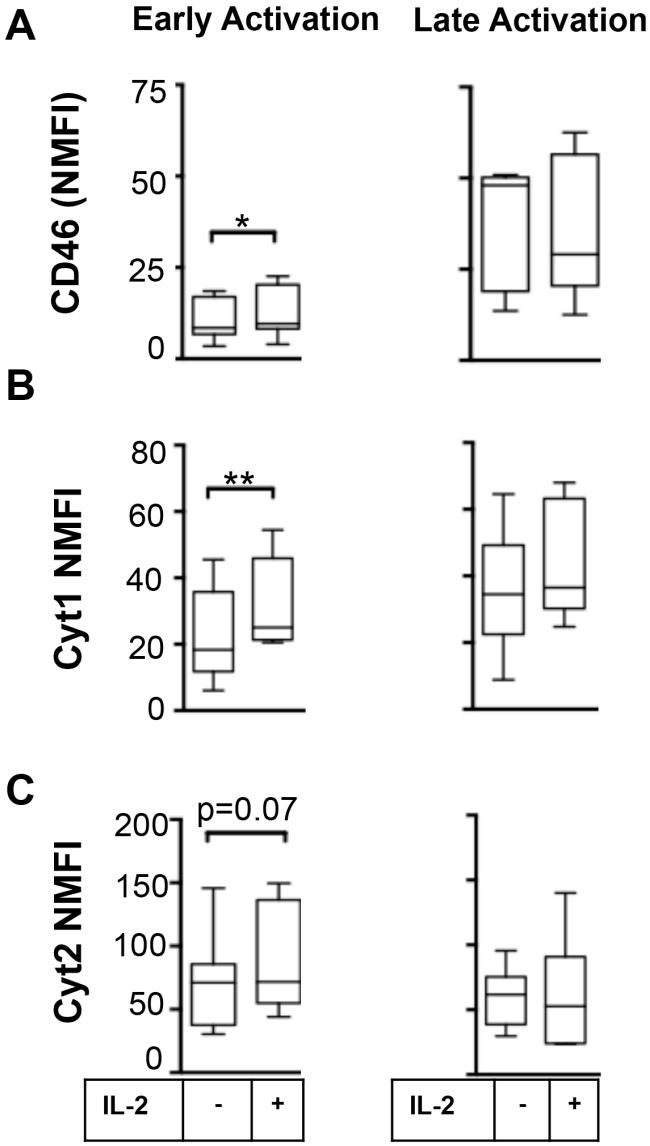


***Figure 3.13: IL-2 modulates CD46 expression.*** *CD46 costimulated cells were activated in the absence or presence of IL-2 (10 U/ml).* ***(A)*** *CD46 expression was analysed by flow cytometry at the early activation (1-2 days stimulation) or late activation (4-5 days stimulation) timepoint (n=5).* ***(B)*** *Cyt1 expression was determined by flow cytometry at the early activation (n =9) and late activation (n=7) timepoint.* ***(C)*** *Cyt2 expression was analysed by flow cytometry at the early activation (n =9) and late activation (n=7) timepoint. NMFI – normalised mean fluorescence intensity. A Wilcoxon test was used for statistical analysis, *p≤0.05, **p≤0.01.*

## 3.5 Discussion

***Surface regulation of CD46 expression***

CD46 expression is increased during activation except upon CD46 ligation (*figure 3.5*). Increased expression is observed upon TCR stimulation alone and is augmented in the presence of CD28 costimulation. Indeed, increased expression was also observed after stimulation with PMA/ionomycin (*figure 3.5D*). Therefore, T cell activation regulates CD46 expression even in the absence of its direct ligation. Upregulation of other costimulatory receptors have been reported upon T cell activation, including, CD28 (Turka et al., 1990), CD278 (ICOS) (Hutloff et al., 1999), CD167 (Dang et al., 2009) and CD27 (Hintzen et al., 1993). Upregulation of NF-$_k$B signalling during T cell costimulation has been well characterised (reviewed in (Kane et al., 2002)) and recently has been shown to increase CD46 expression in breast cancer cells (Cui et al., 2012). Therefore, the upregulation of CD46 during T cell activation could potentially be enhanced by increased NF-$_k$B signalling. The function of upregulated CD46 expression during activation remains to be determined. However, increases in CD46 likely enhance protection of effector T cells against complement during inflammation. It has been established that several tumour cells upregulate complement receptors, including CD46, to shield themselves from complement attack and mediate tumour metastasis (Fishelson et al., 2003, Anderson et al., 2004, Buettner et al., 2007). Moreover, increased CD46 expression may augment the potential for its ligation and signalling during T cell responses. Considering the role of CD46 in promoting IL-10 secretion (Kemper et al., 2003), this could provide a timely negative feedback loop for attenuating T cell responses.

Upon CD46 costimulation in T cells there is a strong downregulation of CD46 (*figure 3.2*) that is concentration (*figure 3.3*) and time-dependent (*figure 3.4*). Downregulation of CD46 in various cell types has been previously reported upon pathogen infection with measles virus (Naniche et al., 1993), adenovirus (Sakurai et al., 2007), *Neisseria gonorrhoea* (Gill et al., 2003), Group A

streptococcus (Lovkvist et al., 2008), gingivalis (Mahtout et al., 2009) and *Helicobacter pylori (Basmarke-Wehelie et al., 2011).* CD46 is also downregulated in apoptotic and necrotic cells (Elward et al., 2005, Hakulinen and Keski-Oja, 2006, Cole et al., 2006). However, this is the first report of CD46 downregulation in primary human $CD4^+$ T cells (Ni Choileain et al., 2011). The role of CD46 downregulation is likely to be multifold depending on the cell type, ligand and mechanism of downregulation. With respect to pathogens, CD46 downregulation can enable pathogen entry and increase cell sensitivity to complement attack (Schnorr et al., 1995). Notably, an increase in CD55 expression, which also protects against complement lysis, was observed in CD46 costimulated T cells (*data not shown*). This upregulation may act to offset the increased complement susceptibility in the absence of CD46.

In T cells, downregulation of CD46 is at least partially due to proteolytic cleavage by MMP/ADAM(s) (*figure 3.6*). CD46 is also cleaved during apoptosis of neuronal and epithelial cells (Hakulinen and Keski-Oja, 2006, Cole et al., 2006), infection of epithelial cells with gingivalis (Mahtout et al., 2009) or *Helicobacter pylori (Basmarke-Wehelie et al., 2011)* and coculture of endothelial cells with Loxoceles venom (Van Den Berg et al., 2002). Proteolysis of surface receptors, such as Notch, ErbB4 and amyloid precursor protein (APP) have important signalling consequences (reviewed in (Parks and Curtis, 2007)). Prior to this research (Ni Choileain et al., 2011) there were no reports that addressed the functional role of cleaved CD46 in cell signalling. However, in *Chapter 4,* a functional role of CD46 proteolysis is demonstrated for the first time in $CD4^+$ T cells. Although, the exact protease(s) involved in the cleavage of CD46 from $CD4^+$ T cells has not been identified, it is likely to involve several, as GM6001 is a broad-spectrum inhibitor of MMP/ADAM(S). Indeed, four proteases have been identified as being involved in the proteolysis of CD46 in other cells types. ADAM10 was implicated in the cleavage of CD46 after its release from apoptotic epithelial cells (Hakulinen and Keski-Oja, 2006). Venom from the spider Loxoceles also induces CD46 shedding from epithelial cells and neutrophils by a

member of the ADAM family (Van Den Berg et al., 2002). Furthermore, MMP3, -8, -9 also contributed to the proteolysis of CD46 from apoptotic neuronal cell lines (Cole et al., 2006). Therefore the expression of MMP/ADAMs *in vivo* could affect CD46 function. Dysregulated expression of MMP/ADAMs has been document in MS patients and could participate in the abnormal function of CD46 in patients (Ni Choileain and Astier, 2011).

Of note, the downregulation of surface CD46 in the presence of plate bound stimulating CD46 antibodies raises the question of whether CD46 downregulation is due to cross-blocking of the stimulating CD46 antibody or stripping of cell surface CD46 from the cell membrane. However, increased soluble levels of CD46 with a decreased $M_w$ upon CD46 costimulation (*figure 3.6C*) and increased surface expression of CD46 in the presence of an MMP/ADAM inhibitor indicated that CD46 downregulation upon CD46 costimulation is at least partially due to proteolysis and is not merely due to the cross-blocking or antibody stripping of cell surface CD46 by the stimulating antibody. Indeed, incubation of an anti-mouse-FITC antibody with cells that had been activated with CD46 antibodies did not result in a positive FITC staining suggesting that no significant levels of stimulating antibody were attached to the cell surface after T cell harvesting (*figure 3.3B*). The concentration of the anti-mouse-FITC antibody used was equivalent to that used for the detection of primary mouse antibodies to Cyt1 and Cyt2 and should have been sufficient to detect significant levels of mouse antibodies attached to the cell surface. Although low levels of cross-blocking or stripping by the stimulating antibody cannot be completely ruled out, these data suggest that CD46 cell surface downregulation is at least partially due to cell surface proteolysis.

***Soluble CD46***

Upon cleavage of CD46, a sCD46 fragment is released (*figure 3.6C*). The exact function of sCD46 during T cell activation remains to be determined. However, it is known that soluble forms of CD46 maintain their complement binding activities (Hakulinen et al., 2004) and recombinant soluble forms of CD46 can

protect tumour cells from complement attack (Gorter and Meri, 1999) and delay hyper acute graft rejection (Christiansen et al., 1996). Proteolytic cleavage of CD46 occurs during cell death and acts to attenuate complement activation (Elward et al., 2005, Cole et al., 2006). In this respect, release of sCD46 may have a similar function during T cell activation, whereby its release from T cells acts to attenuate complement activation and potentially suppress excessive inflammatory responses. Of note, increased levels of sCD46 have been identified in SLE [70], MS (Kawano et al., 1999, Soldan et al., 2001) and cancer patient's sera (Seya et al., 1995) and may reflect increased levels of cell death and/or T cell activation alongside subsequent CD46 proteolytic cleavage. In support of the latter hypothesis, reduced levels of CD46 expression have been reported in lymphocytes isolated from SLE patients and in $CD4^+$ cells from asthma patients (Tsai et al., 2012), whether this directly correlates with increased levels of sCD46 remains to be addressed. Soluble CD46 is not limited to disease states and is found in healthy individuals tears, plasma and seminal fluid (Hara et al., 1992) suggesting a more general role in the immune system. Furthermore, a recent report has identified sCD46 as an anti-microbial agent to *Helicobacter pylori* that functions by blocking urease activity, which is required for bacterial survival. Interestingly, oral administration of a soluble CD46 peptide cleared *Helicobacter pylori* from infected mice and also increased the presence of IL-10 (Basmarke-Wehelie et al., 2011). Therefore, the presence sCD46 in bodily fluids may play a broader anti-bacterial role than previously anticipated. The release of sCD46 from CD46 activated T cells likely has more than one function upon its proteolysis from activated T cells. Further studies would need to be carried out to address the role of sCD46 from T cells and whether its function is dependent on the stimuli for proteolytic release, the tissue in which it is released and if other soluble factors can alter its function.

***Cyt1 and Cyt2 regulation***

The function and signalling pathways of the Cyt1 and Cyt2 isoforms are in the early stages of investigation. Herein, the tight regulation of both Cyt1 and Cyt2

during T cell activation is elucidated. Upon CD46 costimulation there is a timely downregulation of Cyt1 at the early timepoint and Cyt2 at the late timepoint. Upon CD28 costimulation, there is no downregulation of Cyt1 at the early timepoint and it is in fact strongly upregulated. However, at the late timepoint, Cyt2 shows a trend of downregulation, similar to that observed in CD46 costimulation (*summarised in table 3.1*). The downregulation of Cyt1 specifically upon CD46 costimulation suggests that it may play a unique role in CD46 induced responses, whereas, a shared pattern of Cyt2 downregulation may suggest a more general role for Cyt2 in T cell activation/shutdown.

***Table 3.1: Relative change in Cyt1 and Cyt2 expression upon either CD28 or CD46 costimulation compared to CD3/TCR stimulation.***

| | Early activation | | | Late activation | | |
|---|---|---|---|---|---|---|
| **Isoform** | **Cyt1** | **Cyt2** | **Cyt2:Cyt1** | **Cyt1** | **Cyt2** | **Cyt2:Cyt1** |
| **CD3/CD28** | ↑ | ↑ | ↓ | ↑ | - | ↓ |
| **CD3/CD46** | ↓ | - | ↑ | ↓ | ↓ | -/↓ |

The importance Cyt1 and Cyt2 downregulation is suggested by their time-dependent downregulation (*figure 3.10*) and the mechanism by which they are downregulated. Namely, Cyt1 and Cyt2 are cleaved proteolytically by PγS during early activation and late activation, respectively, and this proteolysis, at least partially, accounts for their downregulation (*figure 3.11*). The proteolysis of receptors has important functional consequences. For example, upon PγS proteolytic cleavage of Notch, ErbB4 and the APP a functional ICD with transcriptional activity is released (reviewed in (Parks and Curtis, 2007)). Notably Notch can induce IL-10 secretion in Th1 T cells which is dependent on its proteolysis by PγS (Rutz et al., 2008). The specific downregulation of Cyt1 in CD46 costimulated cells and CD46's function in promoting IL-10 secretion

suggests that CD46's Cyt1 isoform may be an important regulator of CD46 induced IL-10 secretion. Thus, the role of Cyt1 and Cyt2 proteolysis was addressed in *Chapter 4*. Furthermore, if downregulation of Cyt1 and Cyt2 is important for CD46 costimulation, it will be important to assess their downregulation in patients with MS and is discussed in *Chapter 6*.

IL-2 is an important regulator of CD46's regulatory function (Cardone et al., 2010). Slight increases in cell surface CD46 expression and its cytoplasmic tails were observed in the presence of IL-2 (*figure 3.14*). IL-2 signalling is linked with the activation of nuclear NF-$_K$B expression, which has been shown to upregulate CD46 (Cui et al., 2012). IL-2 also induces STAT3 serine phosphorylation (Fung et al., 2003) and STAT3 is known to bind to the CD46 promoter and induce its expression (Buettner et al., 2007). Therefore, at least two pathways downstream of IL-2R activation potentially regulate CD46 expression. Therefore, IL-2 may promote *de novo* CD46 expression upon T cell activation and enhance its potential for signalling. Further investigations are required to determine the exact contribution of IL-2 to the regulation of CD46 signalling pathways.

In summary, a model depicting CD46 cleavage upon CD46 costimulation is shown in f*igure 3.14*. Proteolysis of CD46 by MMP/ADAM(s) and PγS results in the release of sCD46 fragments. Both sCD46 and ICD fragments are likely to play a role in the regulation of inflammation. The function of the ICD fragments will be specifically addressed in *Chapter 4*. Finally, it is important to note that CD3 stimulation alone increases surface CD46 expression and Cyt1 and Cyt2 expression during early activation (*figure 3.2 and figure 3.12*). This suggests that CD46 is inherently involved in T cell activation. The role of T cell activation in regulating CD46 function is addressed in more detail in *Chapter 5*.

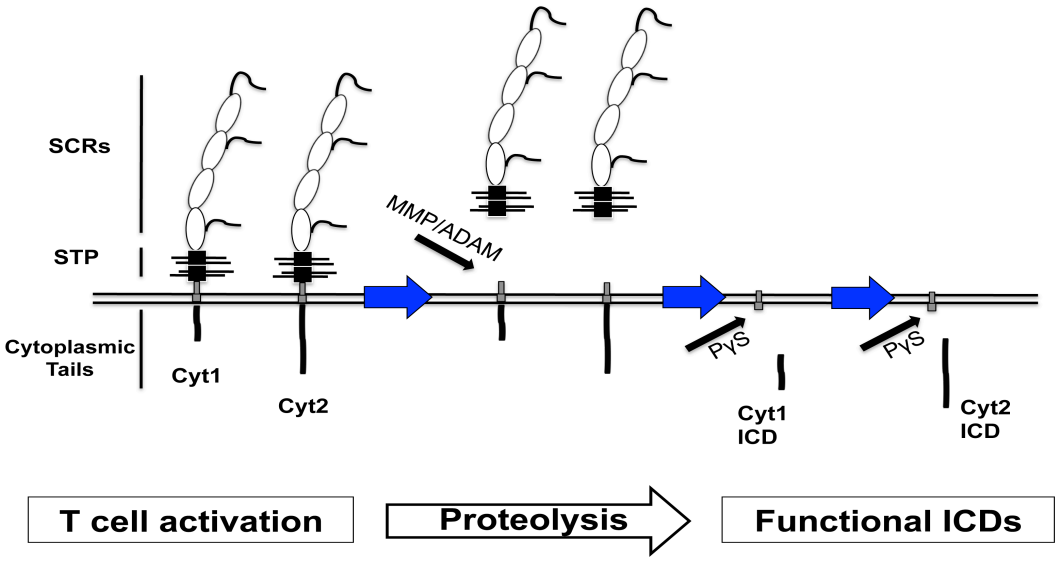


***Figure 3.14: Schematic diagram representing the proteolysis of CD46 upon CD46 costimulation***

### *CD46 Internalisation and degradation*

Downregulation of CD46 is likely to involve internalisation in addition to surface cleavage. Indeed it was previously reported to occur upon CD46 crosslinking in Jurkat T cells and PBMCs in a process similar to macropinocytosis (Crimeen-Irwin et al., 2003). However, internalisation had not been addressed upon CD46 costimulation in primary T cells. In the presence of chloroquine CD46 expression was strongly increased upon CD46 costimulation (*figure 3.8*) suggesting that CD46 is downregulated and degraded though an endosomal/lysosomal pathway. A slight increase was observed as early as 2 hrs post activation (*data not shown*), which suggests that the increase in expression observed is unlikely to be entirely the result of new protein production but rather inhibition of CD46 degradation via the endosomal pathway. Both Cyt1 and Cyt2 sequences contain the YXXL motif, present in the juxtamembrane, which is a known motif for endosomal and lysosomal targeting from the cell surface or trans-golgi network. (Hirano et al., 1996, Yant et al., 1997). YXXL motifs can bind to the clathrin adaptor protein AP-2, inducing internalisation via clathrin coated pits (reviewed in (Boucrot et

al., 2010)). For example, CTLA-4 binds to AP-2, which tags it to the endosomal pathway and marks it for degradation in the lysosome (Shiratori et al., 1997). As CD46 also binds to the adaptor protein AP-2 (Crimeen-Irwin et al., 2003) internalisation of CD46 may also result in lysosome degradation. These data suggest that CD46 surface downregulation upon CD46 costimulation likely involved CD46 internalisation and degradation. Indeed, downregulation of CD46 through the endosomal pathway has been reported previously upon measles virus infection of B cells. Furthermore, CD46 downregulation was required for MHC class II presentation of MV peptides (Gerlier et al., 1994) highlighting a functional role of CD46 downregulation other than degradation.

In the presence of the proteasome inhibitor there was no increase in CD46 expression. In fact there was a dose-dependent decrease upon CD46 expression that was most strongly observed at the surface upon CD46 costimulation *(figure 3.9)*. MG132 inhibits proteasome degradation and also decreases NF-$_k$B expression (Wu et al., 2004). As NF-$_k$B has been shown to positively regulate CD46 expression in cancer cells (Cui et al., 2012), reduced expression of CD46 may be the result of decreased NF-$_k$B. Alternatively, MG132 is capable of inducing apoptosis (Meriin et al., 1998) and may induce CD46 shedding. It has been previously demonstrated that CD46 is shed from the surface of apoptotic cells (Cole et al., 2006). As the effect was more strongly observed at the surface it is likely that cell death may be responsible for shedding of CD46. Colocalisation studies of CD46 and the proteasome/lysosome using confocal microscopy would be useful to shed further light on the mechanism of CD46 degradation. Nonetheless, previous reports and the data acquired here using the lysosomal and proteosomal inhibitors suggest that the lysosomal/endosomal pathway is more likely to be involved in CD46 downregulation and degradation.

Endosomal downregulation is not solely a means of degradation (Miaczynska et al., 2004) and may be important for CD46 signalling. Interestingly, presenilin-1

that forms part of the PγS complex is localised in early endosomes (Lah and Levey, 2000) suggesting that downregulation of CD46 through the endosomal pathway may support its cleavage. Notch signalling is also supported by endocytosis and involves internal sorting to endosomes, the nucleus, lysosomes and possibly signalling endosomes (Fortini, 2009). Moreover, proteolytic cleavage of Notch at the surface and release of its ICD is followed by rapid degradation of the ICD in order to inhibit its potent signalling capacity (reviewed in (Mumm and Kopan, 2000, Fortini, 2009)). Trafficking of CD46, specifically its ICD, could undergo a similar downregulation process. Nonetheless, further experiments are required to confirm the role of endocytosis/lysosome in CD46 trafficking, signalling and degradation.

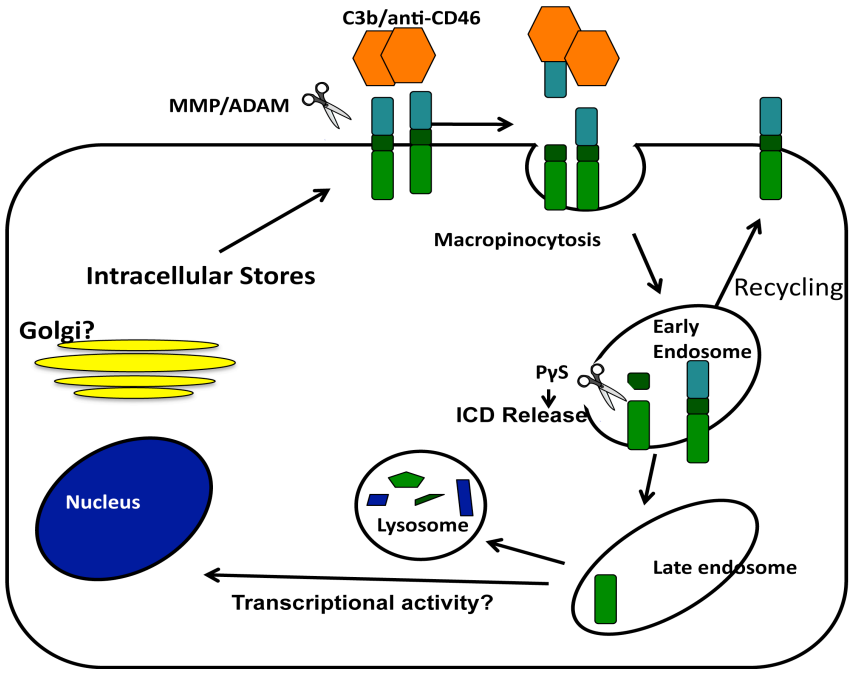


***Figure 3.15: A Schematic representing a potential model of CD46 trafficking and proteolysis in CD4$^+$ T cells.***

## 3.6 Conclusions

- CD46 expression is increased upon T cell activation in the absence of CD46 ligation, and decreased upon CD46 ligation.
- Upon CD46 costimulation Cyt1 is downregulated during early activation and Cyt2 is downregulated during late activation.
- Upon CD28 costimulation, both Cyt1 and Cyt2 are increased during early activation. During late activation, Cyt1 remains upregulated, whereas Cyt2 is decreased.
- Upon CD46 costimulation, CD46 is proteolytically cleaved, at the surface by MMP/ADAM(s) and intracellularly by PγS.
- CD46 downregulation is dependent on a chloroquine-sensitive pathway.

# Chapter 4 : The differential functions of the Cyt1 and Cyt2 isoforms during T cell activation

## 4.1 Introduction

In the previous chapter, a tight regulation of CD46 expression was observed during T cell activation. Moreover, specific expression levels of Cyt1 and Cyt2 were observed throughout T cell activation and T cell shutdown (Ni Choileain et al., 2011) and were suggestive of a distinct functional role for both Cyt1 and Cyt2. Importantly, functional differences between Cyt1 and Cyt2 have previously been reported. For example, in a transgenic mouse model expressing either Cyt1 or Cyt2, Cyt1 expression inhibited inflammation and Cyt2 expression promoted inflammation in a contact hypersensitivity reaction (Marie et al., 2002). Both Cyt1 and Cyt2 have different amino acid sequences (Seya et al., 1999) and it is therefore unsurprising that different cell functions have been observed. For example, there is strong evidence implicating Cyt1 activation with T cell polarity changes (Marie et al., 2002, Ludford-Menting et al., 2002, Oliaro et al., 2006, Ludford-Menting et al., 2011) and autophagy (Joubert et al., 2009), whereas, Cyt2, but not Cyt1, is tyrosine phosphorylated upon CD46 cross-linking (Wang et al., 2000) and *Neisseria gonorrhoeae* infection (Lee et al., 2002). Nonetheless, much work remains to elucidate the signalling cascades of Cyt1 and Cyt2. Determining the specific functions of CD46's isoforms is crucial to understanding of why CD46 function is dysregulated in MS patients. If Cyt1 or Cyt2 activation were specifically responsible for augmenting IL-10 production in healthy controls it would provide greater insights into T cell regulatory function in humans.

In *Chapter 3*, it was also demonstrated that upon CD46 costimulation, CD46 is cleaved by both MMP/ADAM(s) and PγS (Ni Choileain et al., 2011). Cleavage of CD46's ectodomain by MMP/ADAM(s) releases the soluble ectodomain and

the cytoplasmic transmembrane fragment (CTF). The CTF consists of the transmembrane domain and the cytoplasmic tails (either Cyt1 or Cyt2). The CTF can then be further cleaved by PγS, releasing the intracellular domain (ICD), which consists of the cytoplasmic tail only. As mentioned previously, proteolytic cleavage of other type I transmembrane proteins; Notch, ErbB4 and APP result in the release of ICDs with important signalling functions in health and disease (Parks and Curtis, 2007). Interestingly, Notch is involved in T cell differentiation, activation and IL-10 secretion, and the translocation of its ICD to the nucleus facilitates its signalling (Schroeter et al., 1998, Benson et al., 2005, Rutz et al., 2008, Kassner et al.). Therefore, the functional relevance of Cyt1 and Cyt2 CTFs and ICDs were examined during T cell activation. Herein, it is demonstrated that PγS cleavage of Cyt1 facilitates T cell activation whereas uncleaved Cyt2 promotes T cell activation. Furthermore, preliminary evidence suggests that Cyt1 and Cyt2 may also translocate to the nucleus in a time dependent fashion that reflects their cleavage by PγS. Therefore it is possible that both Cyt1 and Cyt2 ICDs, similar to that observed for the Notch ICD, could induce signalling within the nucleus. Furthermore, this thesis highlights CD46 involvement in contracting T cell responses and emphasises its expanding functions in T cell biology.

## 4.2 Aims

1. To determine the function of Cyt1 and Cyt2 in human $CD4^+$ T cells
2. To determine if CD46 proteolytic cleavage is important for CD46 costimulatory function.

## 4.3 Approach

In *Chapter 3* a time-dependent downregulation of Cyt1 during early T cell activation and Cyt2 during late activation was observed. It was also demonstrated that cell surface CD46 was cleaved by MMP/ADAM(s) and that the intracellular tails, Cyt1 and Cyt2, underwent PγS proteolysis. To determine if the proteolysis of Cyt1 and Cyt2 was important for shaping CD46 induced T cell responses, the effect of the MMP/ADAM and PγS inhibitors on T-cell proliferation and IL-10 and IFNγ secretion was assessed.

The experiments outlined above provide a general indication about how proteolysis of Cyt1 and Cyt2 might affect T cell activation. However, it is well known that broad-spectrum inhibitors to MMP/ADAM(s) and PγS have wide-ranging effects and can also inhibit the proteolysis of other receptors. Thus to assess the specific function of the Cyt1 and Cyt2 isoforms and the functional role of their proteolysis during T cell activation, three further approaches were used. Firstly, wild type CTF proteins that consisted of the CD46 transmembrane domain and either the Cyt1 (CTF1) or Cyt2 (CTF2) intracellular tails were constructed (*Nathan Weyand, University of Arizona*) (*figure 4.1*) (Ni Choileain et al., 2011). In *Chapter 3* it was demonstrated that the ectodomain of CD46 is cleaved by MMP/ADAM(s) upon CD46 costimulation. As such, these constructs, that lack the CD46 ectodomain, will help to determine the function of Cyt1 and Cyt2 after the release of their ectodomain during CD46 costimulation. To examine their role, $CD4^+$ T cells were transfected with the vectors expressing CTF1, CTF2 or an empty plasmid called the control vector only (CVO). After transfection, the T cells were activated *in vitro* and their cytokine secretion (IL-10 and IFNγ) was examined to determine if either CTF1 or CTF2 played a specific role in determining the phenotype of CD46 coactivated T cells. The activation state of T cells were also examined by analysing the expression levels of CD25, which is upregulated in human $CD4^+$ T cells upon activation. T-cell proliferation levels were also examined using CFSE incorporation. Analysis of these activation

markers allowed for the role of CTF1 or CTF2 in initiating T cell activation to be examined.

The second approach used to examine the role of Cyt1 and Cyt2 specifically assessed the function of proteolysis by PγS. Mutant CTF1 and CTF2 fragments that were resistant to PγS proteolysis were constructed (*Nathan Weyand, University of Arizona) (Ni Choileain et al., 2011)*. Due to their uncleavable nature they were called UNCL.F1 (uncleavable CTF1) and UNCL.F2 (uncleavable CTF2) (*figure 4.1*). $CD4^+$ T cells were transfected with the vectors expressing UNCL.F1, UNCL.F2 or the wild-type controls CTF1 or CTF2. Following activation, their cytokine and activation state was determined as above. By comparing the function of the cleavable and uncleavable fragments the function of PγS proteolysis could be ascertained.

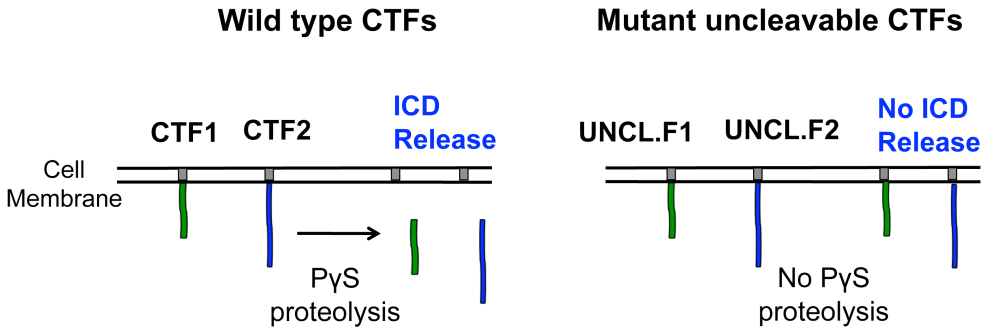


***Figure 4.1: A schematic representation of the wild type and mutant uncleavable CTFs.*** *Wild-type (CTF1, CTF2) and the mutant uncleavable CTFs (UNCL.F1, UNCL.F2) used in this study. CTF – cytoplasmic transmembrane domain.*

The CTF constructs do not have an extracellular domain and therefore could not be specifically activated at the surface. It has been shown previously that CD46 ligation in T cells can alter its cellular location (Ludford-Menting et al., 2011). Thus, surface ligation could have an important role in altering localisation of CD46 upon CD46 costimulation, which may affect its signalling capacities and

function. Therefore the third approach used to investigate Cyt1 and Cyt2 function involved the construction of CD46 fusion proteins. The CD46 ectodomain was replaced with the ectodomain of CD19, a B cell marker that is not expressed on T cells. The CD19 ectodomain was fused to the CD46 transmembrane fragment and cytoplasmic tail, either Cyt1 or Cyt2 (*Joelle Thomas, Université Lyon, France*) (Ni Choileain et al., 2011). These constructs were called CD19-Cyt1 or CD19-Cyt2 and allowed for the specific ligation of either Cyt1 or Cyt2 at the cell surface (*figure 4.2*). Similar to the CTF proteins above, these fusion proteins were transfected into $CD4^+$ T cells and their effect on cytokine secretion and T cell activation was determined after T cell activation. Expression of p-LAT and CTLA-4 were also determined to further elucidate the role of CD46 in T cell activation and contraction. Christian Neumann carried out the vast majority of the work utilising the CD19 fusion proteins during his Masters research project. I contributed to the work by carrying out preliminary experiments, supervising Christian within the lab and through critical review of his contribution to the publication. Christian Neumann performed the data analysis and presentation under the supervision of Dr. Anne Astier.

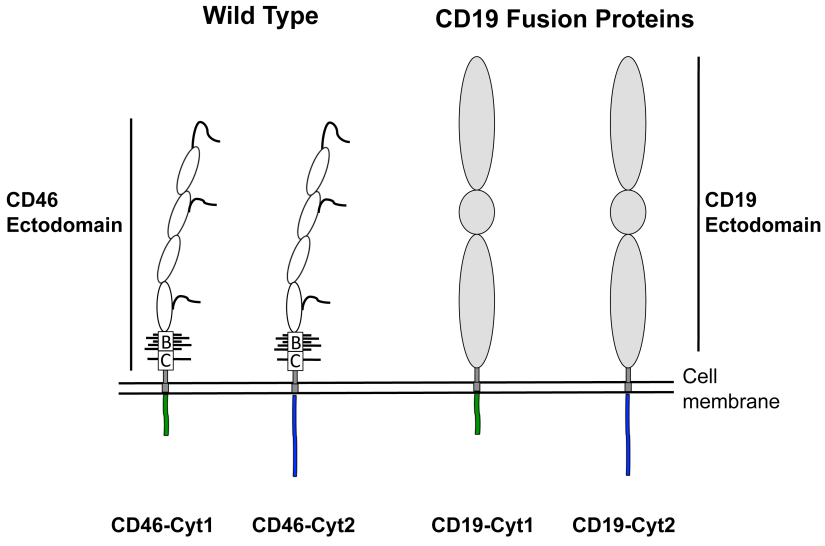


***Figure 4.2: A schematic representation of the CD19 fusion proteins used in this study.*** *(From left to right) Wild type CD46-Cyt1 and CD46-Cyt2. CD19 fusion proteins consisting of the CD19 ectodomain, the CD46 transmembrane fragment and either the Cyt1 (CD19-Cyt1) or Cyt2 (CD19-Cyt2) cytoplasmic tail.*

# 4.4 Results

## 4.4.1 The effect of the MMP/ADAM inhibitor, GM6001, on IL-10 and IFNγ section after CD46 costimulation

In the previous chapter, it was demonstrated that the ectodomain of CD46 was cleaved by MMP/ADAM(s) upon CD46 costimulation. Therefore, in order to determine if cleavage of the CD46 ectodomain was important for its function, the activity of MMP/ADAMs was inhibited using the broad-spectrum MMP/ADAM inhibitor – GM6001. $CD4^+$ T cells were either left unstimulated or stimulated for 5 days with αCD3, αCD3/CD28 or αCD3/CD46 in the presence of GM6001 or DMSO as a control. IL-10 and IFNγ secretion were then determined by ELISA and the ratio of IL-10:IFNγ was calculated. T-cell proliferation was also determined after 3 days of stimulation using $^3$H-thymidine incorporation. In the presence of GM6001, CD46 costimulation showed a trend for decreased IL-10 secretion but had no effect on IFNγ secretion thereby showing a trend for a decreased IL-10:IFNγ ratio. Of note proliferation was not significantly affected in the presence of the inhibitor, although some donors actually showed an increase (*figure 4.3*). Similar results were observed upon CD28 costimulation. However, upon CD28 costimulation a decreasing trend in IFNγ secretion was also observed (*figure 4.3*). Although not significant, these results suggest that inhibition of MMP/ADAMs promoted a decreased ratio of IL-10:IFNγ production upon CD28 and CD46 T cell costimulation. Whether the attenuation of IL-10 secretion involves the specific cleavage of CD46 will be addressed below using the constructs described earlier.

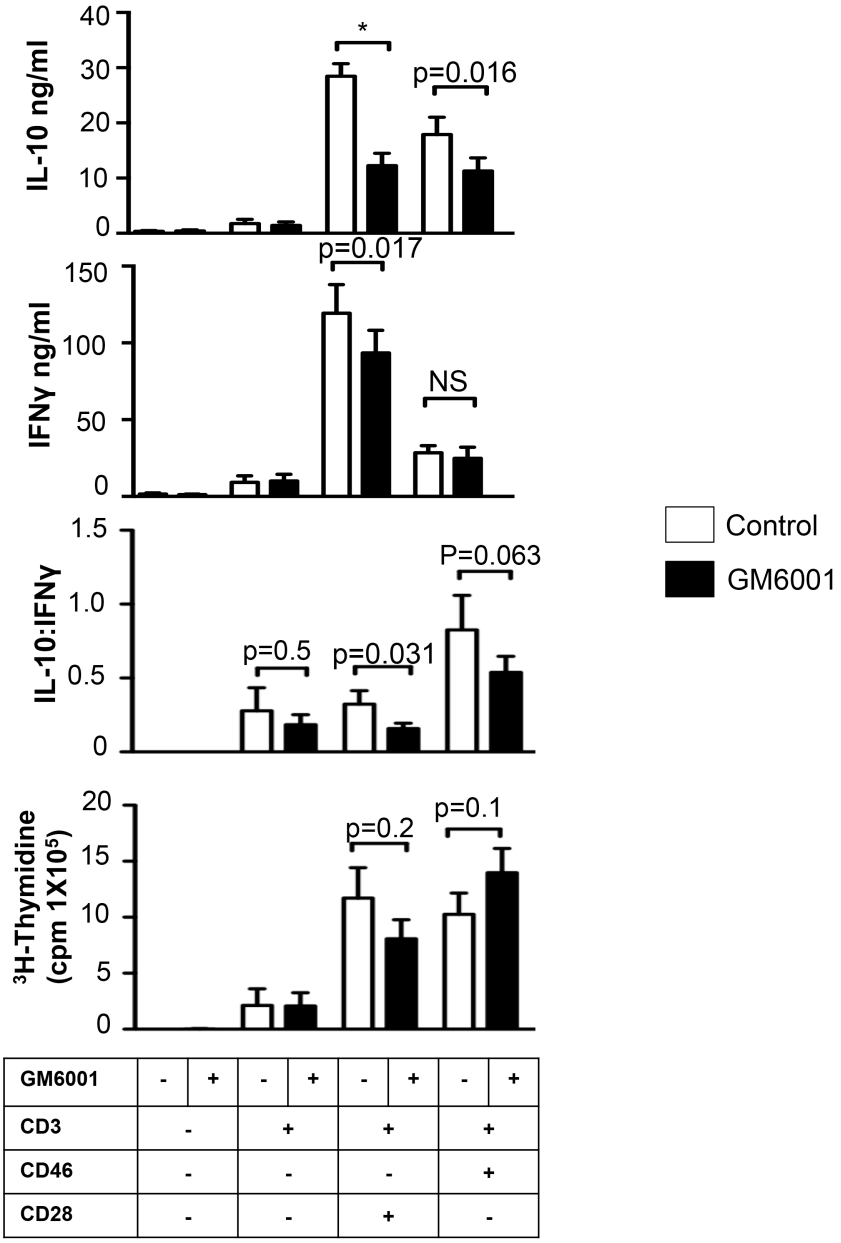


***Figure 4.3: The effect of the MMP/ADAM inhibitor, GM6001, on IL-10 and IFNγ secretion after CD46 costimulation.*** *$CD4^+$ T cells were left unstimulated or stimulated in the presence of αCD3, αCD3/CD28 or αCD3/CD46. The broad-spectrum MMP/ADAM inhibitor, GM6001 (black bars) or DMSO (white bars) was added to each stimulating condition. At day 5, supernatants were collected in order to determine the concentration of IL-10 and IFNγ using ELISA. The ratio of IL-10:IFNγ was also calculated. Proliferation was determined after 3 days stimulation (n=8). A Friedman test and a Bonferroni-corrected Wilcoxon test was used for statistical analysis, *p≤0.01.*

### 4.4.2 The effect of the PγS inhibitor, DAPT, on IL-10 and IFNγ section after CD46 costimulation

In *Chapter 3* it was determined that Cyt1 and Cyt2 underwent proteolysis by PγS on day 2 and day 4 respectively. To determine if PγS proteolysis of the Cyt1 and Cyt2 intracellular tails was important for its function, PγS cleavage activity was targeted using the PγS inhibitor, DAPT. $CD4^+$ T cells were left unstimulated or stimulated with αCD3, αCD3/CD28 or αCD3/CD46 in presence of DAPT or DMSO as a control. The concentration of IL-10 and IFNγ secretion was assessed after 5 days stimulation and the ratio of IL-10:IFNγ was calculated. Proliferation was determined after 3 days of stimulation using $^3$H-thymidine incorporation. In the presence of DAPT, CD46 stimulation resulted in decreased IL-10 secretion compared to DMSO control but had no significant effect on IFNγ or proliferation. There was also no change in the IL-10:IFNγ secretion ratio, suggesting that DAPT minimally affected IFNγ secretion upon CD46 costimulation (*figure 4.4*). Similar results were observed upon CD28 costimulation. Importantly for this study these results indicate that PγS affects IL-10 secretion upon CD46 costimulation and to a lesser extent IFNγ secretion. Whether cleavage of CD46 by PγS is directly involved in CD46 induced IL-10 secretion will be determined below.

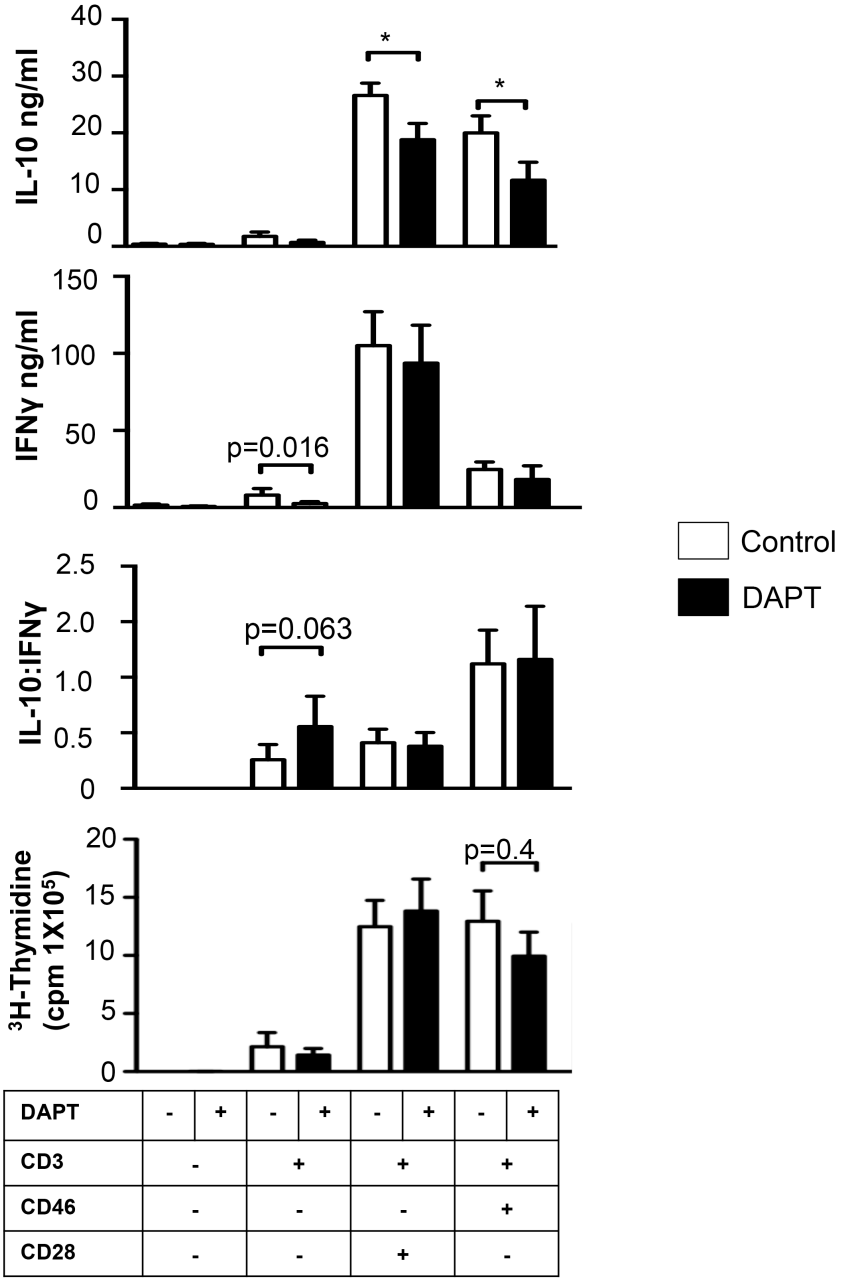


***Figure 4.4: The effect of the PγS inhibitor, DAPT, on IL-10 and IFNγ secretion after CD46 costimulation.*** *$CD4^+$ T cells were left unstimulated or stimulated as indicated. The PγS inhibitor, DAPT (black bars) or DMSO (white bars) was added to each stimulating condition. At day 5, supernatants were collected in order to determine the concentration of IL-10 and IFNγ using ELISA. The ratio of IL-10:IFNγ was also calculated. After 3 days stimulation, proliferation was determined (n=8). A Friedman and Bonferroni-corrected Wilcoxon test was used for statistical analysis, *p≤0.01.*

### 4.4.3 T cell cytokine secretion after transfection with wild-type and mutant CTFs

Surface CD46, Cyt1 and Cyt2 expression is downregulated as a result of proteolysis during CD46 costimulation (*Chapter 3*). Use of broad-spectrum inhibitors indicated that both MMP/ADAM(s) and PγS played a role in CD46 induced cytokine secretion, especially IL-10. However, a direct link between CD46 cleavage and an increased IL-10:IFNγ ratio remained to be determined. Moreover, the effect that CD46 proteolysis had on T cell activation was still unknown. Therefore $CD4^+$ T cells were transfected with the vectors containing different CD46 CTFs, as previously described. Prior to examining the role of these vectors on T cell cytokine secretion and activation, the effect of the empty control vector only (CVO) on cytokine secretion was examined to assess if nucleofection of primary human T cells had any major effects on IL-10 or IFNγ cytokine secretion. $CD4^+$ T cells were transfected with the CVO plasmid and stimulated with αCD3/CD28, αCD3/CD46 or αCD3/CD28/CD46. IL-10 and IFNγ secretion were analysed after 3 days of stimulation using a secretion assay. Cytokine secretion in all conditions was similar to that previously reported (*figure 4.5A*)(Kemper et al., 2003). Upon CD46 costimulation, there was a large percentage of $IL\text{-}10^+$ cells and a low percentage of $IFN\gamma^+$ cells. Conversely, upon CD28 costimulation there was a large percentage of $IFN\gamma^+$ cells and a low percentage of $IL\text{-}10^+$ cells. CD28/CD46 resulted in a moderate percentage of both $IL\text{-}10^+$ and $IFN\gamma^+$ T cells (*figure 4.5A*).

As the CVO appeared to have no apparent effects on normal T cell activation, $CD4^+$ T cells were then transfected with wild type CTF1 and CTF2 to determine their effects on T cell cytokine secretion. After transfection, the T cells were stimulated with αCD3/CD28, αCD3/CD46 or αCD3/CD28/CD46 for 3 days before assessing IL-10 and IFNγ secretion. The percentage of $IL\text{-}10^+$, $IFN\gamma^+$ and $IL\text{-}10^+IFN\gamma^+$ cells was calculated. No significant change in cytokine secretion was observed after transfection with either the CTF1 or CTF2 plasmid (*figure 4.5B, left panel*). Of note there was a large variation of cytokine secretion

observed between donors, that could reflect donor variability, variations in the levels of transfection efficiency and/or variations in the $CD4^+$ T cell subsets obtained from each donor. Importantly for the function of CD46 induced Tregs, after CD28/CD46 costimulation CTF1 increased the ratio of IL-$10^+$:IL-$10^+IFN\gamma^+$ cells (*figure 4.5B, right panel, red box*). *Cardone et al* have demonstrated that CD46 stimulated cells can switch from a Th1-like to a Treg phenotype, transitioning from IL-$10^-IFN\gamma^+$ to IL-$10^+IFN\gamma^+$ and finally to IL-$10^+IFN\gamma^-$ secreting cells (Cardone et al., 2011). Therefore, CTF1 may promote the transition to a more Treg phenotype. No significant affect of the CTF2 transfection was observed under any stimulating condition, further experiments are required to determine the exact function of CTF2 during T cell activation.

Next it was determined if CTF1's promotion of 'IL-10 only' secreting cells was dependent upon PγS cleavage. In order to do this, $CD4^+$ T cells were transfected with the mutant uncleavable plasmids, UNCL.F1 and UNCL.F2 and their effects on cytokine secretion was compared to that of their wild-type counterparts (CTF1 and CTF2). After 3 days of stimulation, the percentage of IL-$10^+$, $IFN\gamma^+$ and IL-$10^+IFN\gamma^+$ cells were determined (*figure 4.5C, left panel*) and the ratio of IL-10:IL-$10^+IFN\gamma^+$ and IL-10:IL-$10^+IFN\gamma^+$ (*figure 4.5C, right panel*) was calculated**.** No significant effect of the uncleavable mutants was observed.

In summary Cyt1 can promote a more regulatory T cell phenotype. Further experiments are required to determine the exact role, if any, of Cyt2 and PγS cleavage in regulating T cell cytokine secretion.

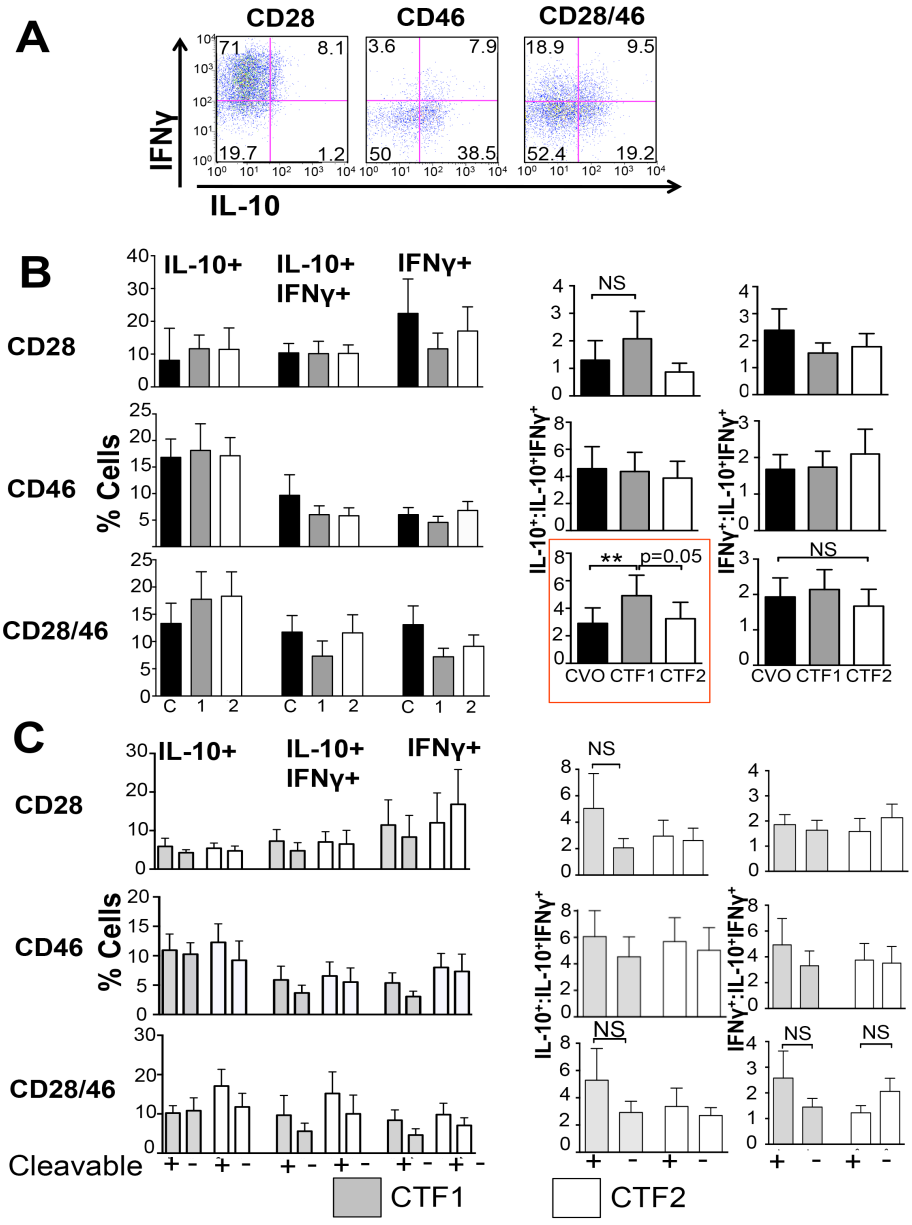


***Figure 4.5: T cell cytokine secretion after transfection with cleavable and uncleavable CTFs. (A)*** *T cells were transfected with the CVO plasmid and stimulated with αCD3/CD28 (CD28), αCD3/CD46 (CD46) or αCD3/CD28/CD46 (CD28/46). IL-10 and IFNγ secretion was determined using a Miltenyi secretion assay after 3 days stimulation.* ***(B)*** *$CD4^+$ T cells were transfected with CVO, CTF1 or CTF2 vectors and stimulated as indicated. IL-10 and IFNγ secretion was determined after 3 days stimulation and the indicated ratios were calculated (n=8). A Friedman test and Bonferroni-corrected Wilcoxon test was used for statistical analysis, **p≤0.003* ***(C)*** *$CD4^+$ T cells were transfected with wild-type CTF1 or CTF2 or the uncleavable mutants UNCL.F1 or UNCL.F2. T cells were stimulated as indicated and the indicated ratios were calculated (n=6). A Friedman test and Bonferroni-corrected Wilcoxon test was used for statistical analysis, NS = non significant..*

### 4.4.4 The effect of CD46 CTF expression on T cell activation and the role of PγS

In the previous section it was demonstrated that CTF1 could affect a T cell's cytokine secretion profile (*figure 4.5)*. In light of their time dependent downregulation it was also hypothesised that Cyt1 and Cyt2 could have different effects on $CD4^+$ T cell activation and proliferation. Therefore the role of the Cyt1 and Cyt2 CTFs in T cell activation and proliferation was assessed. $CD4^+$ T cells were transfected with either an empty control vector (CVO), wild-type CTF1 and CTF2 or the uncleavable mutants, UNCL.F1 and UNCL.F2. Prior to stimulation, T cells were labelled with the proliferation marker, CFSE and then stimulated with αCD3/CD28, αCD3/CD46 or αCD3/CD28/CD46. CD25 expression and proliferation was assessed by flow cytometry after four days of stimulation by flow cytometry. Proliferation was assessed using CFSE staining and the division index was calculated using Flowjo software. The division index measures the average number of cell divisions that a cell in the original population undergoes upon activation. Transfection of T cells with the UNCL.F1 proteins shows a decreased trend of cell proliferation upon CD28, CD46 and CD28/CD46 costimulation compared to CTF1. A significant decrease in CD25 expression was also observed upon CD46 costimulation, which correlated with a decrease in proliferation. For some donors UNCL.F2 also showed a trend for a decrease in proliferation compared to the wild-type CTF2. Of note, although UNCL.F1 transfected cells showed a decrease in CD25 expression that correlated with decreased proliferation, no decrease in CD25 expression was observed for the UNCL.F2 transfected cells. A representative example of proliferation and CD25 expression upon CD28/CD46 costimulation is shown in *figure 4.6A*, and the mean proliferation and CD25 expression of several donors is shown in *figure 4.6B*. These data suggest that cleavage of CTF1 facilitates T cell proliferation as inhibiting CTF1 cleavage attenuates proliferation and CD25 expression. Further experiments are required to assess the role of CTF2 in regulating T cell proliferation.

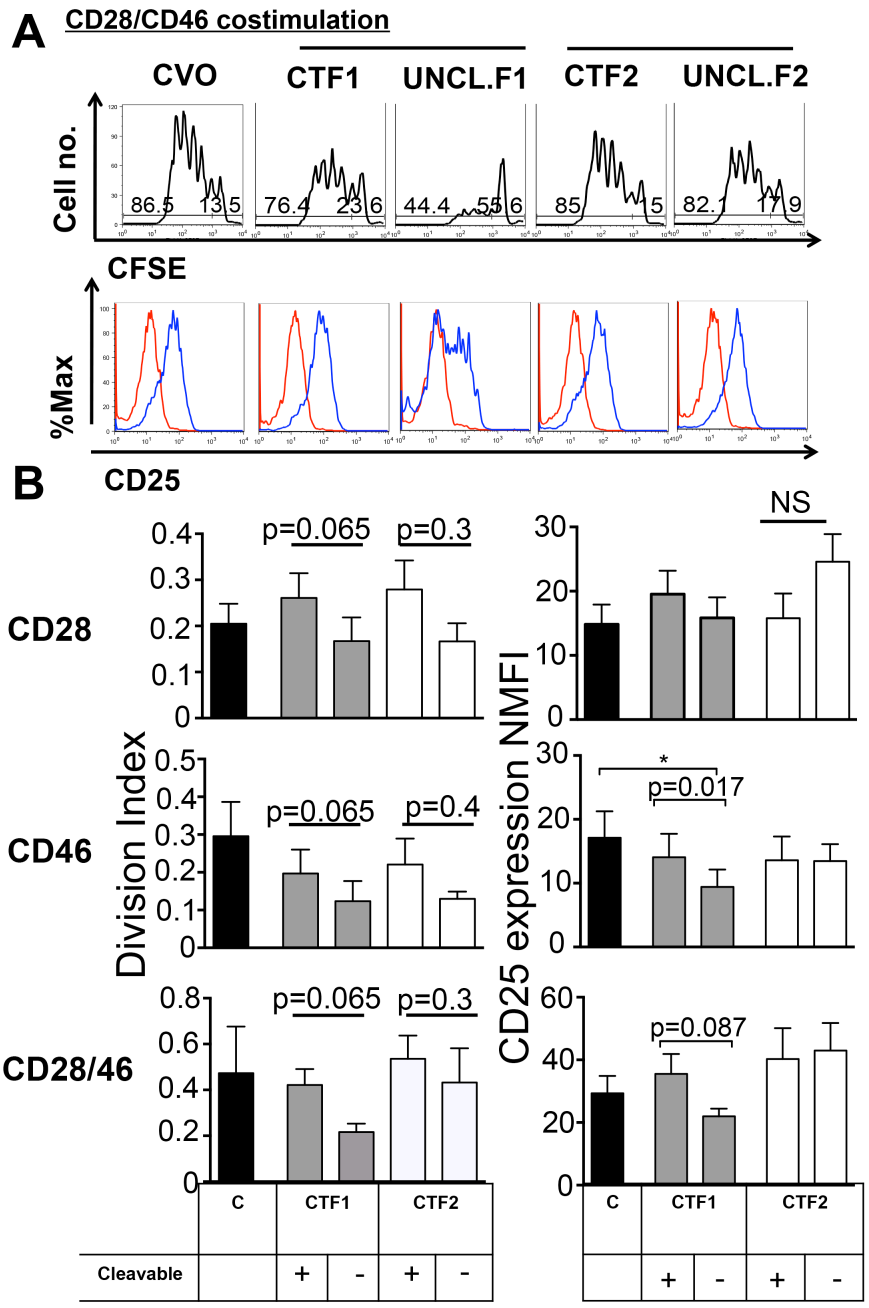


***Figure 4.6: The effect of CD46 CTF expression on T cell activation and the role of PγS.*** *$CD4^+$ T cells were transfected with the control empty plasmid (CVO), wild-type CTF1 or CTF2 (cleavable +) or the uncleavable mutants UNCL.F1 or UNCL.F2 plasmids (cleavable -). Cells were labelled with CFSE before stimulation with αCD3/CD28 (CD28), αCD3/CD46 (CD46) or αCD3/CD28/CD46 (CD28/46). After 4 days stimulation, proliferation was assessed by flow cytometry in addition to CD25 expression.* ***(A)*** *Representative example of CFSE (n=5) and CD25 expression (n=8) upon CD28/CD46 costimulation.* ***(B)*** *The mean division index, used to measure proliferation (n=5) and the mean CD25 expression is shown (n=8). A Friedman test and Bonferroni-corrected Wilcoxon test was used for statistical analysis, *p≤0.008. Error bars = Standard Error Mean.*

### 4.4.5 CD19-CD46 fusion protein expression in HEK293 and T cells

Transfection of $CD4^+$ T cells with different CTF fragments indicates that CTF1 and CTF2 have different effects on T cell cytokine secretion and activation. However, as the CTFs do not contain ectodomains, an additional approach using the CD19-CD46 fusion proteins was adopted. CD19 is not expressed in T cells and would therefore allow the specific ligation of either the Cyt1 or Cyt2 isoform at the cell surface. As described previously, these fusion constructs consisted of the extracellular domain of CD19, a B cell marker, fused to CTF1 or CTF2. These chimeric proteins were called CD19-Cyt1 and CD19-Cyt2 (*figure 4.2*). Firstly, to examine the correct expression of the chimeric proteins, human embryonic kidney (HEK293) cells were transfected with the fusion proteins. HEK293 cells do not express CD19 and are easily transfected, therefore making them a good choice of cells to examine the expression of the plasmids. After transfection, the ectodomain CD19 and intracellular Cyt1 and Cyt2 expression levels were determined by flow cytometry. Expression levels of CD19 after transfection of both CD19-Cyt1 and CD19-Cyt2 were ~35%. Cyt1 expression was specific to the HEK293 cells that were transfected with CD19-Cyt1 and Cyt2 expression was specific to the HEK293 cells that were transfected with CD19-Cyt2 (*figure 4.7A*).

Next, the transfection efficiency of the vectors in primary human T cells was examined. $CD4^+$ T cells were transfected with either the control CVO, CD19-Cyt1 or CD19-Cyt2 vectors and then stimulated with αCD3/CD28, αCD3/CD28/CD19, αCD3/CD46 or αCD3/CD28/CD46. After overnight stimulation CD19 expression levels were determined by flow cytometry. Similar levels of CD19 expression were observed in T cells as for the HEK293 cells (*figure 4.7B*). Of note, upon CD19 ligation there was also a downregulation of CD19 expression that was not observed in any other stimulating condition. This downregulation mirrors the downregulation of CD46 observed upon its ligation and suggests that the CD19-fusion proteins can undergo similar processing to that of wild type CD46. Moreover, CD19 downregulation was not due to masking of

the detection antibody epitopes by the activating CD19 antibody because there was no detection of CD19 upon staining with αIgG-FITC (*data not shown*). In summary, $CD4^+$ T cells could be transfected efficiently with the CD19 fusion proteins and could therefore be used to assess the function of Cyt1 and Cyt2 in $CD4^+$ T cells.

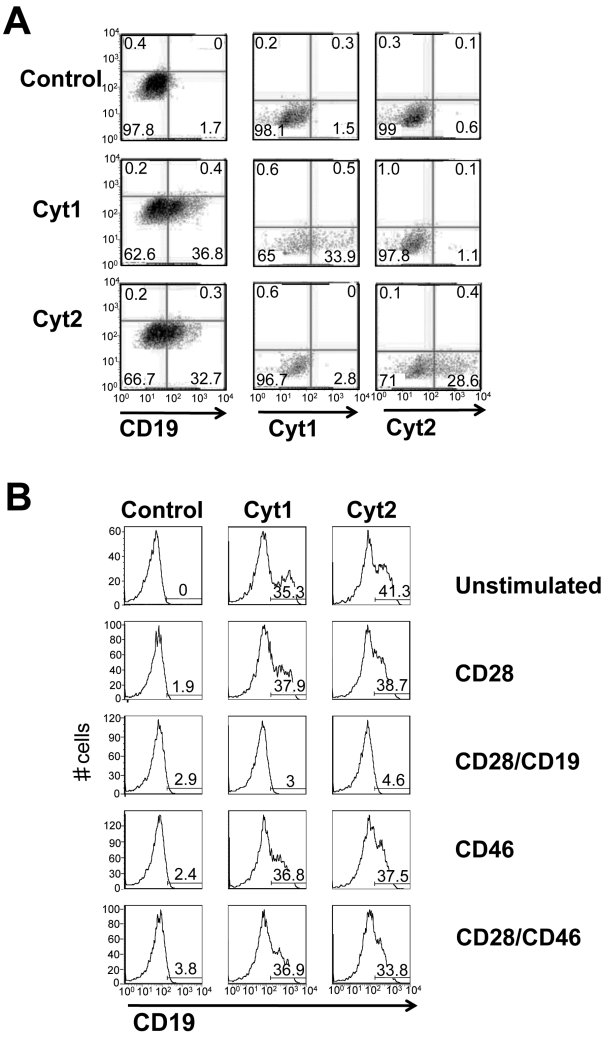


***Figure 4.7: CD19-CD46 fusion protein expression in HEK293 and T cells.*** ***(A)*** *HEK293 cells were transfected with an empty control vector only (Control), CD19-Cyt1 (Cyt1) or CD19-Cyt2 (Cyt2), the next day expression of CD19 (cell surface), Cyt1 and Cyt2 (intracellular) were determined (n=3).* ***(B)*** *$CD4^+$ T cells were transfected with the control, CD19-Cyt1 (Cyt1) or CD19-Cyt2 (Cyt2) vectors. Then T cells were left unstimulated or stimulated with αCD3/CD28 (CD28), αCD3/CD28/CD19 (CD28/CD19), αCD3/CD46 (CD46) or αCD3/CD28/CD46 (CD28/CD46). The next day, expression of CD19 was determined by flow cytometry. Data are representative of 3 independent experiments.*

### 4.4.6 Specific ligation of Cyt1 and Cyt2 differently affected T cell activation

Using the CD19-Cyt1 and CD19-Cyt2 vectors described in the previous section, the function of Cyt1 and Cyt2 could be assessed upon their surface ligation in $CD4^+$ T cells. This additional approach was used to support the findings observed using the CTF plasmids. $CD4^+$ T cells were transfected with CD19-Cyt1, CD19-Cyt2 or a control vector only (CVO). Following transfection, the cells were stimulated with αCD3/CD46, αCD3/CD19, αCD3/CD28 or αCD3/CD28/CD19. In order to determine the role of CD19-Cyt1 and CD19-Cyt2 upon activation, proliferation levels of transfected T cells were examined alongside CD25 expression after 4 days of stimulation. The level of activation was defined as $CFSE^{lo}CD25^+$. Although neither CD19-Cyt1 nor CD19-Cyt2 transfected T cells significantly altered cytokine secretion upon T cell costimulation (*data not shown*), differences in T cell activation could be detected. In all stimulatory conditions, CD19-Cyt1 decreased T cell activation compared to the control transfection, whereas CD19-Cyt2 promoted activation. Therefore, upon their specific ligation Cyt1 and Cyt2 have contrasting roles in regulating T cell activation.

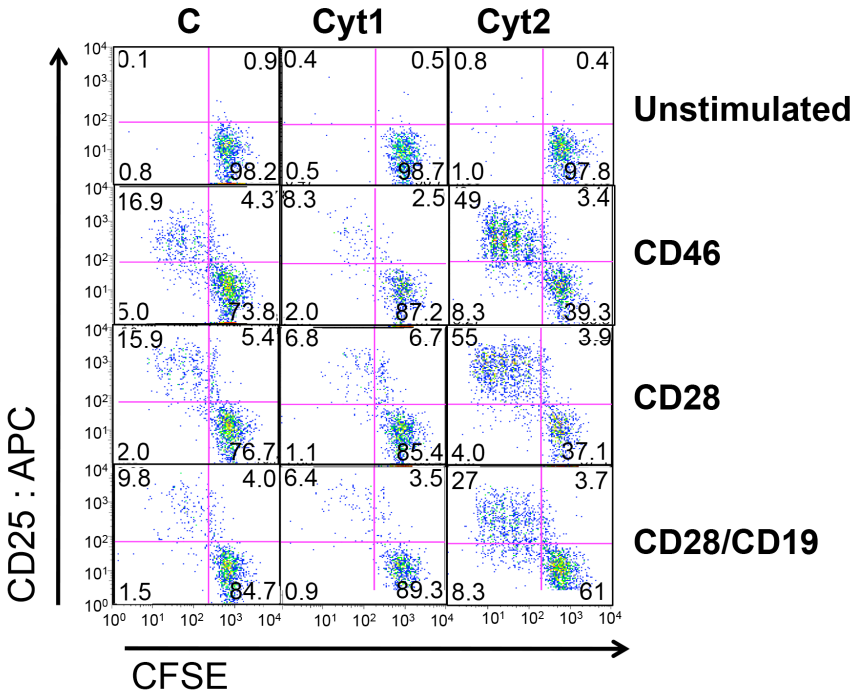


***Figure 4.8: Specific ligation of Cyt1 and Cyt2 differently affected T cell activation.*** *$CD4^+$ T cells transfected with the control vector only (Control), CD19-Cyt1 (Cyt1) or CD19-Cyt2 (Cyt2) and were activated with αCD3/CD28 or αCD3/CD28/CD19. T cells were labelled with CFSE before stimulation with αCD3/CD46 (CD46), αCD3/CD28 (CD28) or αCD3/CD28/CD19 (CD28/CD19). After 4 days stimulation, proliferation was assessed by flow cytometry in addition to CD25 expression (APC). Data are representative of 3 independent experiments.*

### 4.4.7 Specific ligation of Cyt2 increased CTLA-4 expression

In order to further assess the role of Cyt1 and Cyt2 during T cell activation the expression levels of CTLA-4, a T cell coinhibitory receptor, and phosphorylated LAT (p-LAT), an activatory signalling molecule that is phosphorylated upon CD46 activation (Astier et al., 2000), were examined. As above, T cells were transfected with CD19-Cyt1, CD19-Cyt2 or the CVO control. The next day, the cells were either stimulated with αCD3/CD28 or αCD3/CD28/CD19. CTLA-4 expression levels upon CD3/CD28/CD19 costimulation were then compared to CD3/CD28 stimulated cells. There was no change in CTLA-4 expression in the control cells or the CD19-Cyt1 transfected cells. However, CD19-Cyt2 transfected cells, which had been CD3/CD28/CD19 costimulated, increased CTLA-4 expression compared to CD3/CD28 stimulation alone (*figure 4.9A)*.

In order to determine any changes in LAT expression, T cells were either stimulated with CD3/CD28 or CD3/CD28/CD19 in the presence of a cross-linker for 5 min at 37 $^{o}$C and p-LAT expression was determined by flow cytometry. Expression levels of p-LAT upon CD3/CD28/CD19 costimulation were compared with cells stimulated with CD3/CD28 alone. There was no change in p-LAT expression in the control cells or the CD19-Cyt1 transfected cells. However, CD19-Cyt2 transfected T cells stimulated with CD3/CD28/CD19 stimulation induced a decreasing trend in p-LAT expression compared to CD3/CD28 stimulation alone (*figure 4.9B)*. Overall, Cyt2 ligation increases CTLA-4 expression and induces a trend for decreased p-LAT expression. These data suggest that Cyt2 ligation could promote a contraction of T cell responses, however, further experiments are required to confirm these results.

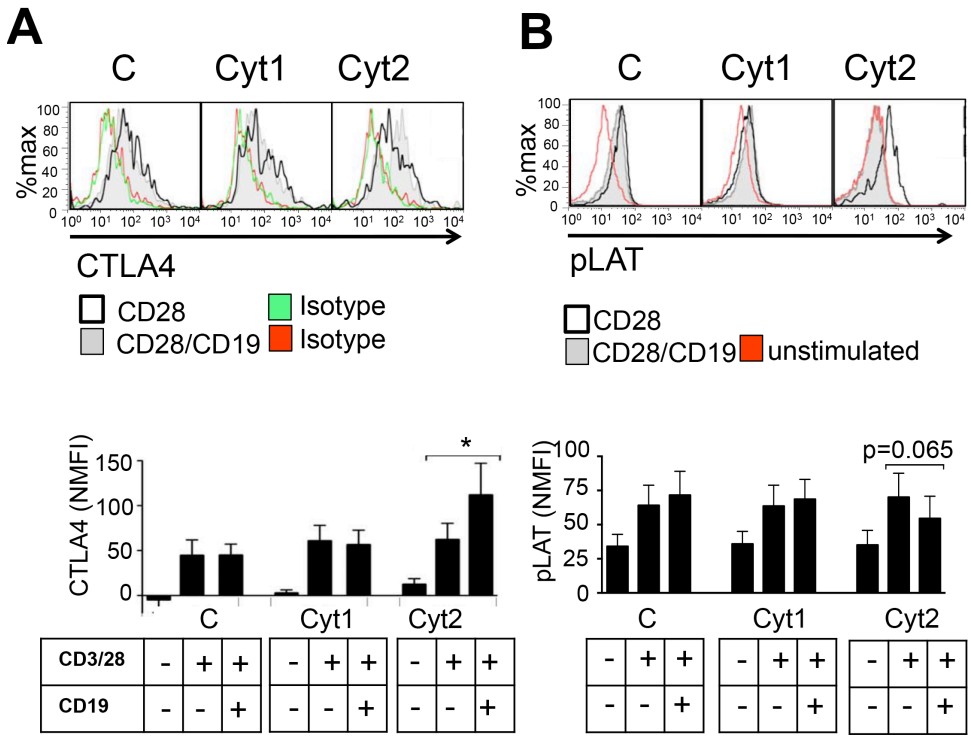


***Figure 4.9: Specific ligation of Cyt2 increased CTLA-4 expression.*** *$CD4^+$ T cells were transfected with the control vector only (C), CD19-Cyt1 (Cyt1), CD19-Cyt2 (Cyt2) and left unstimulated or stimulated with αCD3/CD28 (CD28) or αCD3/CD28/CD19 (CD28/CD19).* ***(A)*** *CTLA4 expression was determined by flow cytometry (Top panel) A representative example of CTLA4 expression. (Bottom panel) The normalised mean fluorescence intensity (NMFI) obtained for 6 independent experiments.* ***(B)*** *Phosphorylated LAT (pLAT) was determined by intracellular flow cytometry staining after 5 min crosslinking. (Top panel) A representative example of pLAT expression. (Bottom panel) mean pLAT expression (NMFI) obtained for 5 independent experiments. A Friedman and Bonferroni-corrected Wilcoxon test was used for statistical analysis, *p≤0.006.*

### 4.4.8 The localisation patterns of Cyt1 and Cyt2 upon T cell activation

Other PγS substrates such as APP, Notch and ErbB4 release ICDs with nuclear transcriptional activity upon proteolytic cleavage (Parks and Curtis, 2007). Both CD46 cytoplasmic domains contain nuclear localization motifs (Seya et al., 1999, Wang et al., 2000) suggesting an ability to translocate to the nucleus and propagate cell signalling. Therefore, the localisation of CD46 Cyt1 and Cyt2 was investigated using confocal laser scanning microscopy. $CD4^+$ T cells were cultured in chamber slides that had been pre-coated with either IgG1 or αCD3/CD46. After 2 or 5 days stimulation the localisation of Cyt1 or Cyt2 was determined. Due to photo-bleaching z stacks could not be taken of the cells, therefore, images were taken at the widest diameter of the nucleus. At day 2, in the absence of CD46 costimulation, Cyt1 and Cyt2 were strongly localised at the periphery with smaller amounts of intracellular staining. Of note, the peripheral Cyt1 staining was less uniform than Cyt2. Upon CD46 costimulation, Cyt1 but not Cyt2 expression is downregulated at the periphery and appears to localise in the vicinity of the nucleus. At day 5, unstimulated cells maintained uniform Cyt2 staining at the periphery; however, upon CD46 stimulation there was an increase in Cyt2 staining within the vicinity of the nucleus *(figure 4.10)*. These results suggest that there may be relocalisation of both Cyt1 and Cyt2 in activated cells from the periphery to the nucleus. Relocalisation appears to be temporal and corresponds to the downregulation of Cyt1 during early activation (day 2) and Cyt2 during late activation (day 5) (*Chapter 3*). These data suggest that proteolytic cleavage of Cyt1 and Cyt2 could promote transcriptional functions and that they are regulated by time. However, more extensive studies need to be carried out with increased cells numbers, z stacks and colocalisation studies.

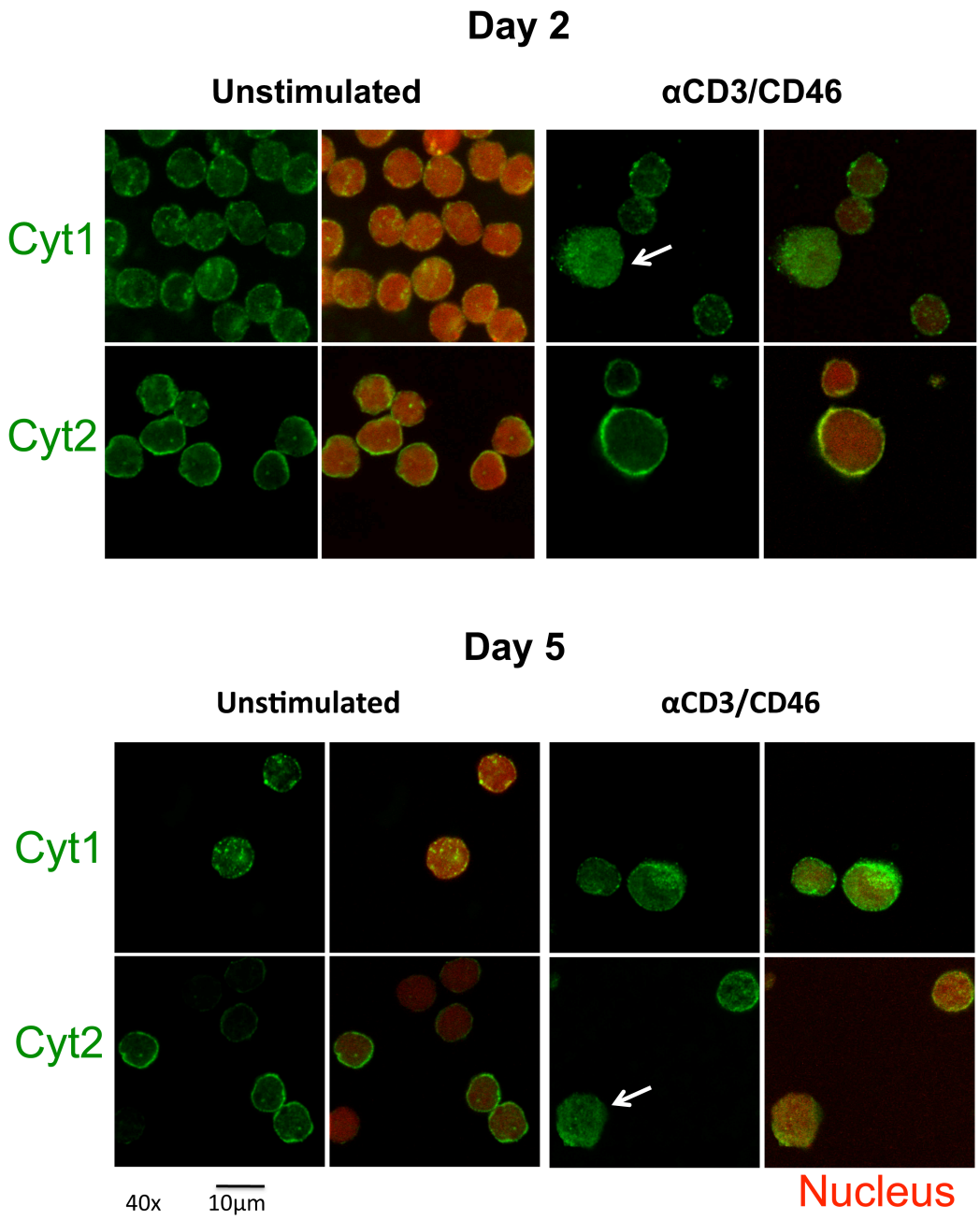


***Figure 4.10: The localisation patterns of Cyt1 and Cyt2 upon T cell activation.*** *$CD4^+$ T cells were left unstimulated (IgG1) or stimulated with αCD3/CD46 and for 2 or 5 days before confocal analysis. Cyt1 and Cyt2 (green signal), nucleus counterstain (red signal). Images were acquired with the Zeiss510meta and exported to ImageJ (n=1.).*

## 4.5 Discussion

The T cell phenotype induced by CD46 can be pro-inflammatory or anti-inflammatory (Kemper et al., 2003, Sanchez et al., 2004). However, little is known about the signalling pathways that CD46 utilises for T cell regulation. Moreover, it is known that soluble factors such as IL-2, PGE-2 and vitamin D influence the type of immune responses initiated following CD46 costimulation (Cardone et al., 2010, Kickler et al., 2012) (*and manuscript in press*). However, little is known about how CD46 isoforms regulate CD46 function in human T cells. This chapter expands on the known functions of CD46's isoforms in T cells. In light of the results described in this chapter, the role of the Cyt1 and Cyt2 isoforms in T cell activation and cytokine secretion is discussed below.

***Cyt1 and IL-10 secretion***

CD46 induced IL-10 secretion is fundamental to its ability to suppress bystander proliferation (Kemper et al., 2003). Furthermore *figure 4.3 and figure 4.4* demonstrate that MMP/ADAM(s) and PγS play a role in promoting IL-10 production during CD46 costimulation. However, it remained to be determined if Cyt1 and/or Cyt2 specific proteolysis was involved. Therefore, determining if Cyt1 or Cyt2 could specifically promote IL-10 secretion would greatly enhance our understanding of CD46's regulatory capacity. In this regard, transfection of $CD4^+$ T cells with the wild type CTF1 constructs promoted a higher ratio of IL-$10^+$:IL-$10^+$IFN$\gamma^+$ secreting cells. Conversely, the CTF2 construct had no positive effect in promoting an increased ratio of IL-10 only secreting cells (*figure 4.5 and figure 4.8A*). Whilst preparing a manuscript describing these results (Ni Choileain et al., 2011), *Cardone et al* also demonstrated that Cyt1 expression in the Jurkat T cell line was responsible for CD46 induced IL-10 secretion and promoted the switch from IL-$10^+$IFNγ+ to IL-10 only producing cells (Cardone et al., 2010). Therefore, Cyt1 acts to promote IL-$10^+$ cells that do not produce IFNγ and may be an important target in understanding why there is defective IL-10 secretion from CD46 costimulated T cells in MS patients (Astier et al., 2006).

It remained to be determined if CD46 proteolysis was directly involved in promoting IL-10 secretion. To determine if PγS cleavage of CD46 was important for IL-10 secretion, $CD4^+$ T cells were transfected with either the uncleavable UNCL.F1 or UNCL.F2 constructs. Transfection of T cells with the mutant uncleavable UNCL.F1 and UNCFL.F2 had no significant effect on the cytokine secretion profile (*figure 4.5C*). Failure to observe a significant result in this experiment is likely due to variations in transfection efficiency or a low n-number given the high level of variability. Further experiments are required to confirm the role of Cyt1 and Cyt2 PγS cleavage in T cells. Nonetheless it is interesting to note that Notch1 has the capacity to induce PγS dependent IL-10 secretion in Th1 cells (Rutz et al., 2008, Kassner et al.) and therefore Notch could play a role in CD46 induced IL-10 secretion. PγS cleavage of Notch requires the prior cleavage of its ectodomain by MMPs. Thus inhibition of Notch cleavage with the inhibitors could explain the decreased IL-10 secretion observed upon CD28 and CD46 costimulation (*figure 4.4*). Whether PγS of CD46 plays an additional role in promoting IL-10 secretion remains to be determined.

***Cyt1 proteolysis and T cell activation***

In *Chapter 3*, it was demonstrated that Cyt1 undergoes PγS cleavage and downregulation after 1-2 days of CD46 costimulation. UNCL.F1, a mutant CTF1 construct that inhibited PγS cleavage showed a strong trend of reduced T cell activation,, compared to wild type CTF1 and this trend correlated with a significant decrease in CD25 expression (*figure 4.6*). This indicated that cleavage of CTF1 was important for T cell activation. Moreover, under both CD46 and CD28 costimulating conditions, CD19-Cyt1 expression decreased T cell activation compared to cells transfected with the CVO (*figure 4.8B*). This could correlate with the uncleavable UNCL.F1 data since in the absence of CD19 ligation cleavage of CD19-Cyt1 is predicted not to occur. Therefore in the absence of Cyt1 proteolysis by PγS, Cyt1 inhibits T cell activation. Conversely, cleavage of Cyt1 during T cell activation facilitates normal T cell activation.

*Russell et al* have previously shown that Cyt1 can compete with the immunological synapse (IS) for polarity. Prior to T cell activation, cross-linking of CD46, by antibodies or measles virus proteins (which bind CD46), strongly reduced IS formation and decreased T cell activation and function (Oliaro et al., 2006). Cyt1 inhibited IS formation by recruiting lipid rafts and the microtubule-organising centre (MTOC) to the CD46 ligation site (Oliaro et al., 2006, Ludford-Menting et al., 2011). On a signalling level, Cyt1 interacts via its FTSL domain with the scaffold proteins; Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC) and discs, large homolog 4 (DLG4) (Joubert et al., 2009). Both GOPC and DLG4 contain protein binding motifs called PDZ domains (Ludford-Menting et al., 2002) that regulate T cell morphology and polarity during immunological synapse formation and T cell migration (Ludford-Menting et al., 2005). Therefore, there is strong evidence to support the role of Cyt1 in T cell polarity changes. Importantly, this competition with the TCR for T cell polarity was specific to Cyt1 and was not observed for Cyt2 (Oliaro et al., 2006, Ludford-Menting et al., 2011). In line with these findings *Marie et al* reported that Cyt1 transgenic mice reduced $CD8^+$ T cell activation in a contact hypersensitivity model (Marie et al., 2002). Thus, in the absence of CTF cleavage, as is the case for the UNCLF.1 and CD19-Cyt1, (in the absence of CD19 ligation) there may be sustained signalling that competes with the IS for polarity. Herein, a model for Cyt1 proteolysis is proposed where proteolysis of Cyt1 prevents Cyt1 from diverting the axis of polarity away from the immunological synapse. By inhibiting Cyt1 cleavage it is possible that Cyt1 maintains signalling and polarity competition that diverts MTOC and lipid rafts away from the IS, thereby attenuating T cell activation.

***Cyt2 and T cell proliferation***

Upon CD46 costimulation, Cyt2 is initially upregulated during activation but is downregulated after 4-5 days of stimulation. This time-dependent downregulation of Cyt2 was at least partially the result of PγS cleavage and suggested that during late activation Cyt2 proteolysis had a distinct function (*Chapter 3*). Due to the

upregulation of Cyt2 during early activation and the timing of Cyt2 downregulation, it was hypothesised that proteolysis of Cyt2 could facilitate the contraction of T cell responses. In order to determine if this was the case, T cells were transfected with the wild-type cleavable CTF2 fragment and the uncleavable UNCL.F2 fragment. However the uncleavable CTF2 mutant showed no increase in proliferation compared to the wild-type fragment and in some donors actually decreased proliferation as measured by the division index. Of note unlike CD19-Cyt1 transfected cells, CD19-Cyt2 did not decrease CD25 expression (*figure 4.6*). Interestingly, transfection of T cells with the full length CD19-Cyt2 protein increased T cell activation compared to the control transfection group under all stimulating conditions indicating that full length Cyt2 plays a role in promoting T cell activation. Notably, upon the ligation of CD19-Cyt2, in the presence of CD28 costimulation, the percentage of activated T cells decreased compared to cells activated with CD28 costimulation alone (*figure 4.8B*). As surface ligation of the CD19 chimeric proteins also resulted in its surface downregulation (*figure 4.7B*), it could indicate that Cyt2 is required in its full-length form and is perhaps required at the cell membrane to promote proliferation. This model is supported by evidence that CD19-Cyt2 ligation, which decreases CD19 surface expression, decreases p-LAT and promotes a T cell phenotype more susceptible to shutdown via the upregulation of CTLA4 expression (*figure 4.9*). It would be interesting to address the question of whether cleavage of Cyt2's ectodomain is the key factor in regulating Cyt2's function because inhibiting PγS's proteolysis of Cyt2 does not restore Cyt2's function in promoting proliferation. Indeed, although no significance was reached, the MMP inhibitor GM6001, increased proliferation in the majority of donors upon CD46 costimulation. This was not observed upon CD28 costimulation (*figure 4.3*) or in the presence of the PγS inhibitor, DAPT (*figure 4.4*). Therefore, full-length Cyt2 promotes T cell activation. Further experiments are required to determine if the loss of this function is due to alterations in cellular location or cleavage of its ectodomain.

Regulated T cell responses not only require correct T cell activation but also a timely termination of the response (Marrack et al., 2010). Surface downregulation of CD46 has previously been reported during cell death of Jurkat, a human T cell line, and neuronal cells (Elward et al., 2005, Cole et al., 2006). For example, CD46 is released from apoptotic Jurkat cells, in a caspase-dependent manner (Elward et al., 2005) and soluble levels of CD46 also increase in parallel with apoptotic caspase activity in epithelial cells (Hakulinen and Keski-Oja, 2006). Indeed, apoptosis induced in epithelial cells by the pathogen *Streptococcus,* which binds CD46, also results in CD46 shedding (Lovkvist et al., 2008). Therefore the release of cell surface CD46 has previously been associated with cell death. This is the first report that suggests a link between Cyt2 processing and T cell shut down. Whether proteolysis of Cyt2 plays a direct role in inducing apoptosis of T cells or if apoptosis directly promotes Cyt2 cleavage remains to be elucidated. Of note, apoptosis has previously been shown to initiate PγS cleavage of the type 1 transmembrane protein ErbB4 thereby promoting the disassembly of epithelial adherence junctions. Moreover, PγS cleavage of ErbB4 required prior proteolysis by an MMP/ADAM and its cleavage was caspase dependent (Marambaud et al., 2002). This example draws clear parallels to CD46 processing, which also undergoes caspase dependent ADAM cleavage in epithelial cells during apoptosis (Hakulinen and Keski-Oja, 2006). Neisseria infection of epithelial cells also induces CD46 shedding (Gill et al., 2005) and PγS proteolysis (Weyand et al.). Therefore, CD46 and especially Cyt2 may be inherently linked to cell death and likely to play an important role in the contraction of T cell responses.

***The localisation and signalling of the intracellular domains***

The question that remains to be addressed is the localisation of the ICDs after γ-secretase cleavage. Their localisation likely has important consequences for CD46 signalling and may offer further avenues of investigation when assessing dysfunctional CD46 signalling in MS patients. PγS cleavage of Notch results in relocalisation of its ICD to the nucleus and is crucial for it signal transduction.

Moreover, the transport to the nucleus of the Notch ICD involves endocytosis at the surface, endosomal trafficking and importantly cessation of signalling upon degradation, all of which are fundamental to the regulation of its signalling (Bray, 2006, Fortini, 2009). These facts raise questions about CD46's ICDs cellular trafficking. For example, is endosomal trafficking important for their function and do they translocate to the nucleus? In *Chapter 3*, it was demonstrated that CD46 is downregulated through the endosomal pathway suggesting that CD46 cleaved fragments may also traffic through the endosomal pathway. Their subsequent degradation could be a key factor in attenuating CD46 signalling. In an attempt to discover the localisation of Cyt1 and Cyt2 upon activation, preliminary confocal studies were carried out that suggest potential relocalisation of both Cyt1 and Cyt2 to the nucleus. Their translocation to the vicinity of the nucleus coincided with the defined timepoint of PγS cleavage- Cyt1 translocation during early activation and Cyt2 translocation during late activation (*figure 4.10*). Despite it's preliminary nature, it is worth emphasising that both Cyt1 and Cyt2 contain nuclear localisation motifs suggesting a biological significance (Seya et al., 1999, Wang et al., 2000). Thus, trafficking of the Cyt1 and Cyt2 ICDs may involve the endosomal pathway, which facilitates their translocation to the nucleus and subsequent degradation. However, more work would need to be carried out to determine it CD46 endosomal trafficking, localisation and degradation is important for CD46's function.

It is also important to note that ICDs may also form signalling complexes within the cytoplasm. One possible signal complex may involve E-cadherin and the actin cytoskeleton. E-cadherin is an important adhesion molecule in epithelial cells and like CD46 is a substrate for γ-secretase cleavage (Marambaud et al., 2002). CD46 binds E-cadherin and α-E-catenin a molecule that links E-cadherin to the actin cytoskeleton (Cardone et al., 2011). Interestingly, in epithelial cells γ-secretase cleavage results in the release of E-cadherin from actin cytoskeleton and initiates the disassembly of adherens junctions (Marambaud et al., 2002). CD46 is also cleaved by γ-secretase in epithelial cells upon its ligation (Weyand et al.).

Furthermore, CD46 ligation on epithelial cells also augmented the permeability of epithelial monolayers and induced epithelial cell proliferation and wound healing (Cardone et al., 2011). Given the proximity of CD46, E-cadherin, E-catenin and PγS in epithelial cells there may be a role for PγS cleavage of CD46 in the proliferation of epithelial cells, similar to that observed in T cells. Moreover, considering the role of Cyt1 in T cell proliferation and alterations in polarity, it would be interesting to determine if the role of CD46 in wound healing was specific to Cyt1. This also raises the question of whether Cyt1 PγS proteolysis releases the ICD from the signalling complex attached to the actin cytoskeleton in T cells, thereby inhibiting Cyt1 from competing with the IS for polarity and facilitating normal T cell activation.

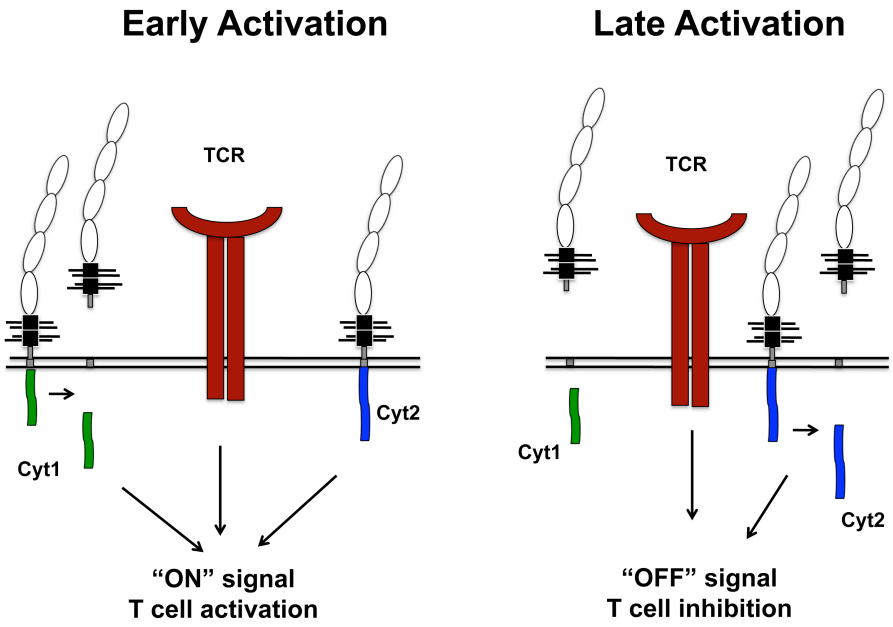


***Figure 4.11: Proposed model of Cyt1 and Cyt2 function in T cell activation.***

To conclude, proteolytic cleavage of CD46 supports CD46 dynamic responses and demonstrates for the first time that proteolytic cleavage of Cyt1 and possibly Cyt2 regulates T cell activation (*figure 4.11*). Aberrant regulation of Cyt1 and Cyt2 cleavage could partially explain the defect of CD46 function in autoimmune

diseases. Further studies, addressing the localisation and interaction of CD46's ICDs will shed light on important regulatory mechanisms related to CD46's diverse signalling functions.

## 4.6 Conclusions

- Similar to that observed after CD28 costimulation, inhibition of MMP/ADAM(s) and PγS cleavage, using broad-spectrum inhibitors, decreases IL-10 secretion upon CD46 costimulation.
- Cyt1 expression increases the ratio of IL-$10^{+}$ secreting cells compared to those that secrete both IL-10 and IFNγ.
- Inhibiting Cyt1's PγS proteolysis decreases CD25 expression, which correlates with a decreasing trend in proliferation.
- Full length Cyt2 promotes T cell activation. Upon the specific ligation of CD19-Cyt2 there is an increase in CTLA-4 expression in addition to a decreasing trend in p-LAT expression.

# Chapter 5 : T cell activation and Glycosylation Regulate CD46 Expression and Function

## 5.1 Introduction

Protease cleavage of receptors plays an influential role in receptor activity by regulating surface expression and propagating signals (Katzmann et al., 2002, Parks and Curtis, 2007, Fortini, 2009). C*hapter 4* demonstrated that cleavage of CD46 by metalloproteinases/ADAM(s) occur upon T cell activation and plays a role in initiating and possibly in terminating T cell responses (*Chapter 5*). Given that the cleavage of CD46 alters CD46 function and T cell responses, the regulation of CD46 surface expression was further investigated. At a basic level T cell stimulation is a two-step process involving TCR ligation and a co-stimulatory signal. It was shown in *Chapter 3* that TCR stimulation increased CD46 cell surface expression and suggested that there was an inherent link between CD46 and T cell activation. Therefore, the role of the TCR in regulating CD46 surface expression was investigated.

CD46 like the majority of immune receptors is glycosylated (*figure 5.1*) (Ballard et al., 1988). Glycosylation has previously been shown to alter the surface expression of important immunological receptors, such as the TCR, CD4, CD45 and CTLA-4 (Pulido and Sanchez-Madrid, 1992, Tifft et al., 1992, Demetriou et al., 2001, Partridge et al., 2004, Chen et al., 2009). Glycosylation can either promote or attenuate surface retention of receptors. For example, O-glycosylation can inhibit protease degradation of proteins while they are expressed on the surface or in endosomes (Reddy et al., 1989, Maryon et al., 2009) and may also facilitate receptor protease cleavage (Gasbarri et al., 2003).

Of note, activation of T cells induces changes in glycosylation (Piller et al., 1988, Galvan et al., 1998, Comelli et al., 2006, Clark and Baum, 2012) that can alter T cell activation and functions (Demetriou et al., 2001, Lau et al., 2007, Chen et al., 2009, Kuball et al., 2009, Clark and Baum, 2012). For example, T cells modulate the glycosylation of CD45, a cell surface glycoprotein, during development and activation (Clark and Baum, 2012), and alterations of its N- and O- glycans can modulate both CD45 clustering, TCR signalling and T cell apoptosis (Nguyen et al., 2001, Amano et al., 2003, Clark and Baum, 2012). We therefore proposed that TCR activation would not only affect CD46 expression but also its state of glycosylation. Furthermore, it was predicted that these changes in glycosylation could regulate CD46 expression and function. CD46 contains both N- and O-glycosylation attachments sites (*figure 5.1*). Therefore, the contribution of each glycan subtype to CD46 function was assessed. In this chapter we identify the TCR as a key regulator of CD46 surface expression and glycosylation. This thesis also provides evidence for a role of CD46 glycans in CD46 surface downregulation and function during T cell activation.

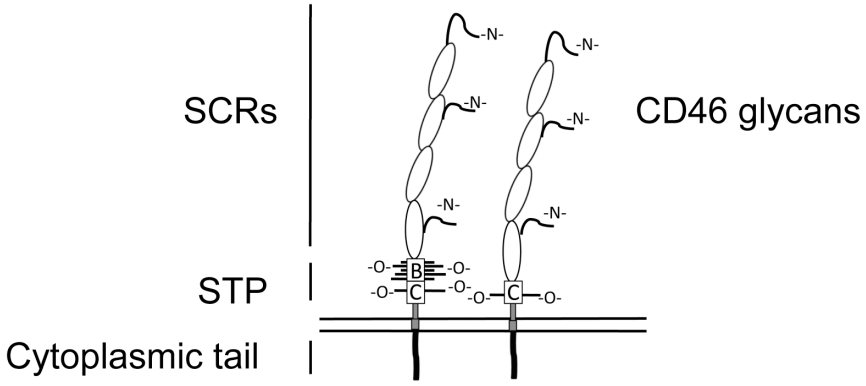


***Figure 5.1: Schematic representation of CD46 with N- and O-glycans.*** *Both the BC and C isoforms of CD46 are represented. SCR - short consensus repeats, STP - serine- threonine-proline-rich domain of CD46.*

## 5.2 Aims

1. To determine the role of T cell activation (by the TCR) in regulating CD46 cell surface expression.

2. To determine if the TCR alters CD46 glycosylation state upon activation.

3. To determine the role of N- and O-glycans in CD46 cell surface expression and T cell function.

## 5.3 Approach

CD46's role as a costimulatory molecule is dependent on TCR activation (Astier et al., 2000). As CD46's proteolytic cleavage is important for T cell activation (*Chapter 4*) (Ni Choileain et al., 2011)), the role of the TCR in regulating CD46 downregulation and proteolysis was investigated. Firstly, CD46 cell surface expression was monitored at various time-points upon CD46 ligation in the absence or presence of αCD3 stimulation. Secondly, intracellular levels of CD46 were examined to identify any retention of CD46 intracellularly. Finally, sCD46 was measured in the supernatants to determine if any changes in CD46 surface expression were the result of proteolytic cleavage.

Given the role of glycosylation in regulating cell surface expression of other receptors, it was also determined if TCR activation induced changes in CD46 glycosylation. Firstly, the $M_w$ of CD46 following T cell activation was monitored in unstimulated or TCR stimulated T cells. $M_w$ changes often reflect differences in glycosylation. Thus, if a difference in $M_w$ was observed between unstimulated and stimulated T cells, it would then be determined if the change in $M_w$ reflected changes in glycosylation. In order to do this, unstimulated and TCR activated T cells would be stripped of their glycans using deglycosylating enzymes (glycanases). If deglycosylation eliminated the differences in $M_w$ observed between unstimulated and stimulated cells, then it would be concluded that changes in $M_w$ were the result of glycosylation alterations upon activation.

CD46 contains 3 potential sites for N-glycosylation and several sites for O-glycosylation (*figure 5.1*). In order to determine if either N-glycans or O-glycans were specifically involved in CD46 downregulation and function, two different approaches were adopted. Firstly, broad-spectrum N- and O-glycosylation inhibitors were used to assess the effect of N- and O-glycans on CD46 cell surface downregulation. In order to determine the effect of N- and O-glycosylation on T cell activation, CD69 expression was determined in the

presence or absence of the inhibitors. IL-10 and IFNγ secretion was also examined to determine if N- or O-glycosylation could affect CD46 function.

As broad-spectrum inhibitors affect the glycosylation of all proteins, this initial approach was supported by the expression of several mutants of CD46, lacking specific attachment sites for N- and O-glycosylation (*section 5.4.3*, *table 5.1*). These mutants were stably expressed in Chinese hamster ovary (CHO) cells that lack endogenous CD46 (Maisner et al., 1996, Liszewski et al., 1998) and were kindly provided by the Atkinson lab (University of Washington, St Louis, MO). CHO cells expressing these mutants of CD46 were cultured in the presence or absence of αCD46 to determine the effect of specific N-glycans or O-glycosylation on CD46 downregulation. T cells were then transfected with the mutant CD46 plasmids and cell surface expression was assessed upon CD46 costimulation. Finally, T cells that had been transfected with the mutant plasmids, were activated in the presence or absence of CD46 costimulation, and cytokine secretion and proliferation were analysed. Together, the data obtained from these experiments would identify the function of specific glycans in CD46 downregulation and function.

# 5.4 Results

## 5.4.1 TCR activation promotes cleavage of CD46

The expression levels of CD46 were first determined in the presence of TCR engagement in order to determine the role of TCR signalling in regulating CD46 expression. Purified human $CD4^+$ T cells were left unstimulated or activated in the presence of αCD3, αCD3/CD46 or αCD46. Cell surface expression of CD46 was examined by flow cytometry at 1 hr, 2 hrs, 3 hrs and 24 hrs. CD46 ligation in the presence of TCR stimulation induced a downregulation of CD46 surface expression as early as 1 hr after stimulation and was more strongly downregulated at 3 hrs and 24 hrs compared to CD46 ligation alone (*figure 5.2A*). TCR induced downregulation of CD46 is αCD3 dose dependent in Jurkat cells (*data not shown* – Dana Photiou, Honours Student). Decreased staining was not due to masking of the epitopes by the stimulating CD46 antibodies, as there was no staining present with an α-mouse IgG1 FITC secondary antibody (*Chapter 3*). Analysis of CD46 intracellular staining also revealed a decrease in CD46 expression following αCD3/CD46 or αCD46 ligation compared to unstimulated cells (*figure 5.2B*). To determine if the increased downregulation of CD46 expression observed upon αCD3/CD46 stimulated cells compared to αCD46 ligation was due to increased proteolytic cleavage of CD46, T cells were cultured under the stimulating conditions described above and the supernatants were collected after 24 hrs of stimulation. The early timepoint of 24 hrs was chosen to minimise differences in protein quantity as a result of proliferation. Soluble CD46 (sCD46) was immunoprecipitated from the supernatants and analysed by western blot (*figure 5.2C*). Upon αCD3/CD46 stimulation there was a band present at ~55 kDa that corresponds to the size of cleaved sCD46 (Van Den Berg et al., 2002, Hakulinen et al., 2004, Hakulinen and Keski-Oja, 2006). This band is absent or reduced upon αCD46 ligation alone, indicating that TCR ligation increased cleavage of CD46 at the surface. Therefore, CD46 ligation in the presence of TCR activation increases CD46 surface downregulation and this was at least partly due to increased proteolytic cleavage of CD46.

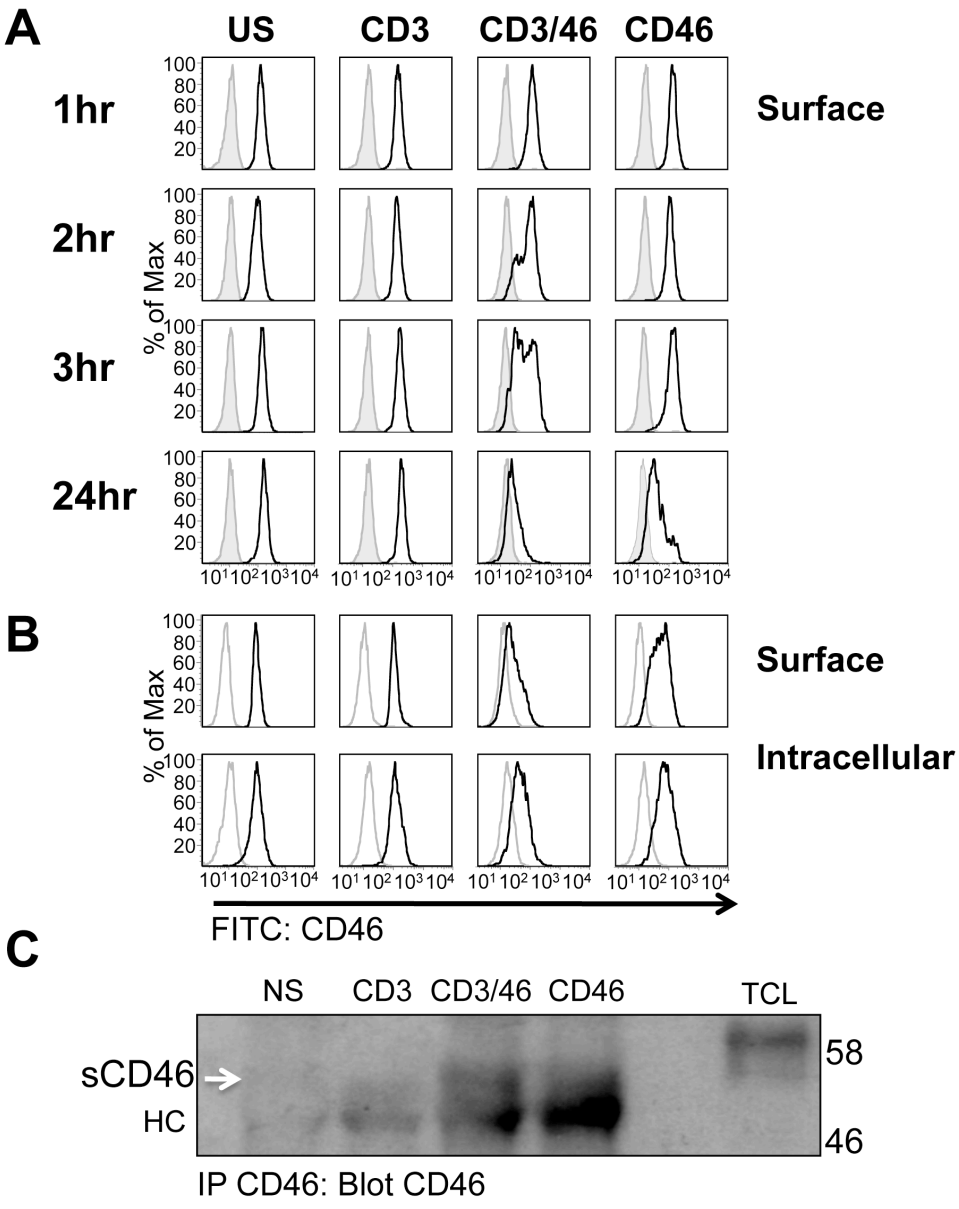


***Figure 5.2: TCR activation promotes the cleavage of CD46.*** *$CD4^+$ T cells were left unstimulated (US) or stimulated with αCD3, αCD3/CD46 or αCD46 alone.* ***(A)*** *Samples were collected at the indicated time points to analyse CD46 surface expression by flow cytometry (representative of 3 independent experiments).* ***(B)*** *After 24 hrs stimulation, surface CD46 and intracellular expression was determined by flow cytometry (representative of 3 independent experiments).* ***(C)*** *Immunoprecipitation (IP) of soluble CD46 (sCD46) in supernatant after 24 hrs stimulation of $CD4^+$ T cells. HC (IP IgG heavy chain), TCL (Total cell lysate) (representative of 2 independent experiments).*

### 5.4.2 The $M_w$ of CD46 is decreased upon TCR activation

T cell activation induces alterations in T cell glycosylation that are important for T cell function (Demetriou et al., 2001, Lau et al., 2007, Chen et al., 2009, Kuball et al., 2009, Clark and Baum, 2012). Therefore, it was determined if TCR activation induced changes in CD46 glycosylation. First, the $M_w$ of CD46 was examined by SDS-PAGE and western blotting from T cells that had been left unstimulated or activated with αCD3, αCD3/CD46 or αCD46. There was a systematic decrease of ~2 kDa in the $M_w$ of CD46 upon TCR activation (αCD3). This decrease is observed after 2 days and 4 days activation (*figure 5.3A*). These donors expressed both the BC and C CD46 isoforms and both isoforms had decreased $M_w$ upon activation. This decrease in $M_w$ was also observed upon CD46 co-stimulation (*figure 5.3A*) and CD28 co-stimulation (*figure 5.3B*). After T cells were stimulated with an increasing dose of αCD3 (0.2-25.0 μg/ml), there was a dose dependent decrease in the $M_w$ of CD46 (*figure 5.3C*). Nonetheless, TCR activation is required to induce a decrease in the $M_w$ of CD46, as CD46 ligation in the absence of CD3 stimulation does not affect its $M_w$ (*figure 5.3D*). Therefore, TCR activation induced a dose dependent decrease in the $M_w$ of CD46 that was also observed upon CD28 and CD46 costimulation. These data indicate that glycosylation of CD46 may change upon activation and this is further investigated below.

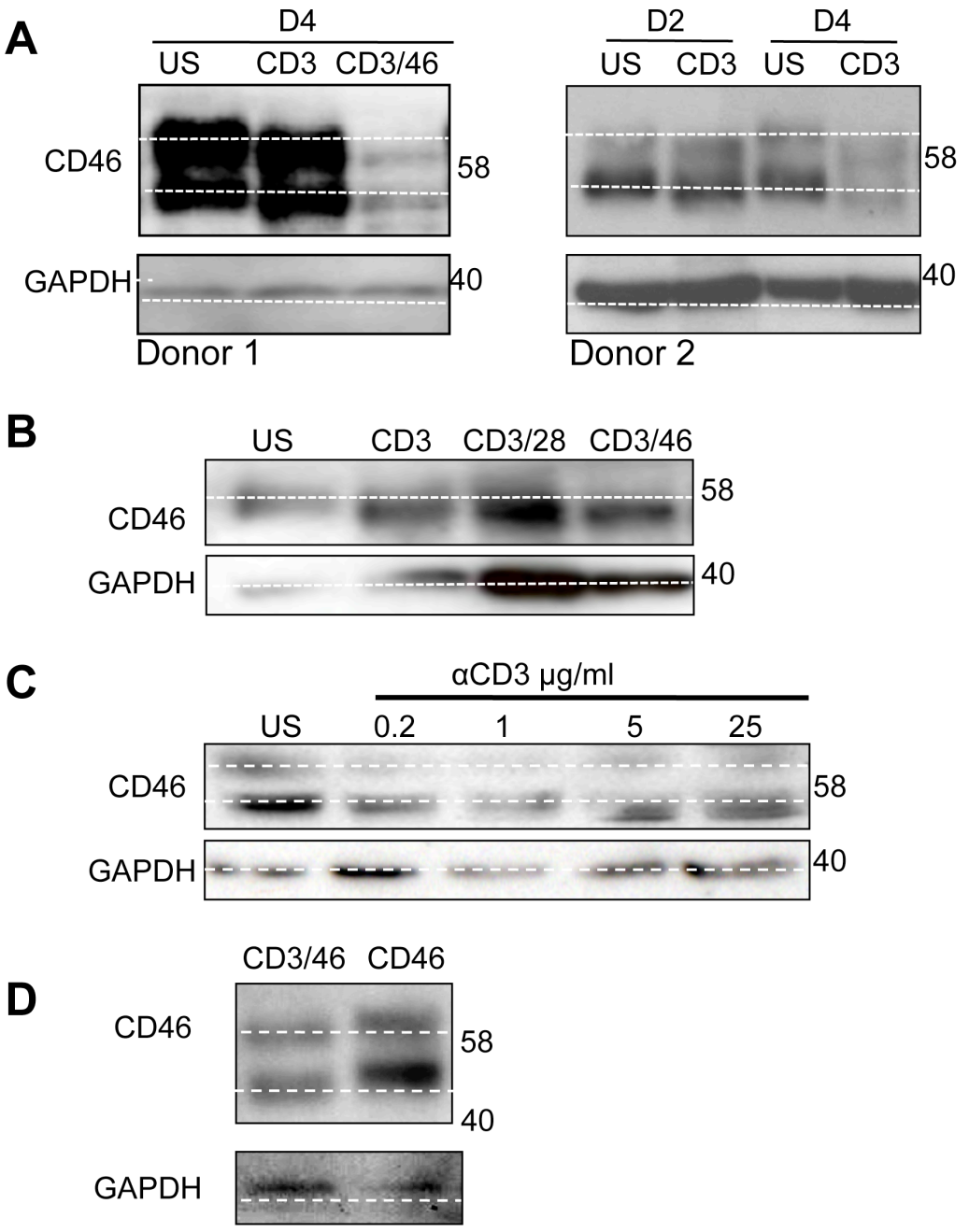


***Figure 5.3**: **The $M_w$ of CD46 is decreased upon T cell activation. (A)** (Left panel) T cells were left US or stimulated with αCD3 alone or in the presence of αCD46 (CD3/46) for 4 days. (Right panel) T cells were left US or stimulated with αCD3 for 2 or 4 days. (representative of 5 independent donors) and the $M_w$ of CD46 was determined using SDS-PAGE. **(B)** T cells were stimulated with αCD3/CD28 (CD3/28) for 2 days in addition to the stimulating conditions described in (A). (Representative of 3 independent donors). **(C)** T cells were left US or stimulated with increasing doses of αCD3 (0.2 – 25 μg/ml) for 5 days. **(D)** T cells were stimulated overnight with αCD3/CD46, or αCD46. (Representative of 4 independent donors). GAPDH was used as a loading control (A-D).*

### 5.4.3 TCR stimulation induces decreased glycosylation of CD46

As CD46 contains three N-glycosylation sites and multiple O-glycosylation sites (*figure 5.1*), it was possible that the reduction in CD46's $M_w$ observed in *figure 5.3* was the result of deglycosylation upon TCR activation. In order to investigate this, T cells were left unstimulated or activated with αCD3 for five days. T cells were lysed and a small fraction of the lysate was used to assess the $M_w$ of CD46 by SDS-PAGE, to confirm that a decrease in $M_w$ occurred upon activation (*figure 5.4A*). The remaining total cell lysate was used to immunoprecipitate and deglycosylate CD46. If deglycosylation eliminated the differences in $M_w$ observed between unstimulated and stimulated cells, then it would be concluded that changes in $M_w$ were the result of glycosylation alterations upon activation. The immunoprecipitate was denatured and split into two equal aliquots, one was untreated and the other aliquot underwent O- and N-deglycosylation using the ProZyme Enzymatic deglycosylation kit and ProZyme prO-Link Extender™. Deglycosylation was carried out according to the manufacturers protocol. In brief, the kits contain N-glycanase PNGase F®, sialidase A, O-glycanase® (ProZyme Enzymatic deglycosylation kit) and β(1-4)-specific galactosidase and β-N-acetylglucosaminidase (ProZyme prO-Link Extender™). N-glycanase® cleaves the N-glycan linkage between the first sugar of the N-glycan core and the asparagnine residue of the glycosylated protein. This enzyme functions for all known human N-glycans. A similar enzyme for removing all O-glycans does not exist and several enzymes must be used to remove commonly expressed O-glycans on human T cells. Therefore the combination of O-glycanase®, sialidase A™, β(1-4)- specific galactosidase and β-N-acetylglucosaminidase was used to remove the O-glycans. Untreated and glycosidase treated samples were incubated overnight at 37 $^{o}$C and the following day CD46 $M_w$ was determined by SDS-PAGE. In the absence of glycanase treatment a decrease in CD46 $M_w$ was observed upon αCD3 stimulation (*figure 5.4B*) similar to that observed in the total cell lysate (*figure 5.4A*). Upon glycanase treatment the $M_w$ of CD46 was reduced to 42 kDa in both the unstimulated and αCD3 stimulated immunoprecipitates. This is the reported $M_w$ of deglycosylated CD46 (Cervoni et al., 1992, Maisner et al., 1994, Hara et al.,

1998). These data suggested that the decrease in $M_w$ observed in activated T cells is due to a decrease in glycosylation. Overall therefore, T cell activation induces a decrease in the $M_w$ of CD46 either as a result of deglycosylating existing CD46 or through the induction of *de novo* CD46 protein translation with a lower level of glycosylation.

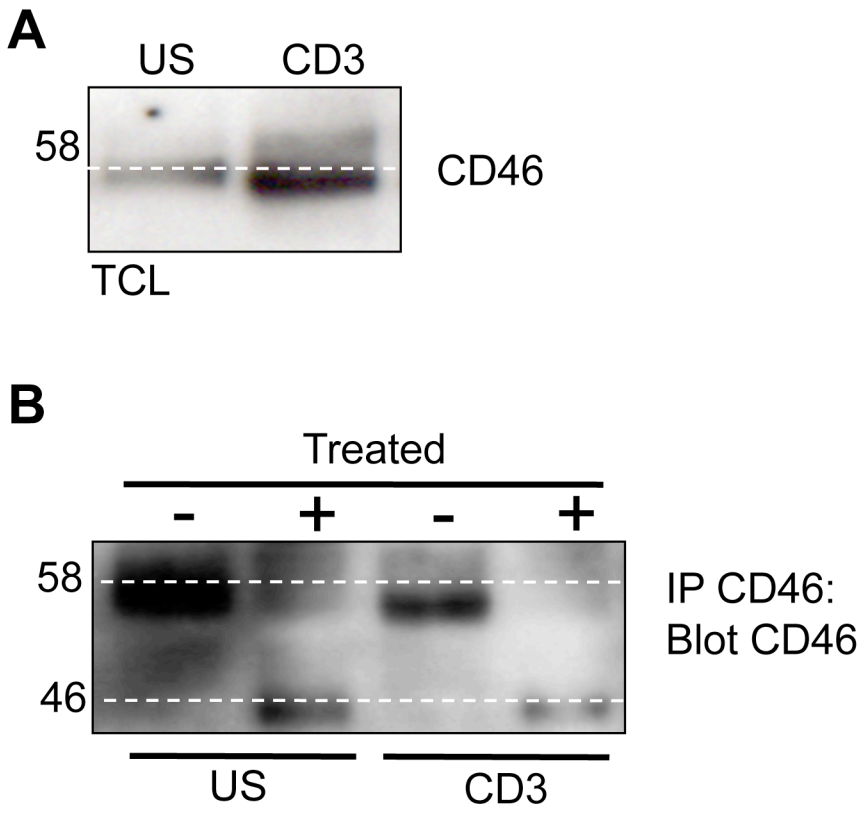


***Figure 5.4: A decrease in the $M_w$ of CD46 upon TCR activation is the result of decreased CD46 glycosylation***. *$CD4^+$ T cells were left unstimulated (US) or activated with αCD3 (CD3) for 5 days. Cells were lysed and SDS-PAGE was carried out on the* ***(A)*** *total cell lysate (TCL) and* ***(B)*** *CD46 immunoprecipitate (IP). The CD46:IPs were untreated (-) or treated with N-glycanase, O-glycanase, Sialidase and the ProZyme extender kit to remove N and O-glycans (+), (representative of 2 independent experiments).*

### 5.4.4 Broad-spectrum inhibitors of O- and N-glycosylation have opposite effects on cell surface CD46 expression

T cell activation induced a decrease in the level of CD46 glycosylation. Glycosylation alters the surface expression of immunological receptors, such as the TCR, CD4, CD45 and CTLA-4 (Pulido and Sanchez-Madrid, 1992, Tifft et al., 1992, Demetriou et al., 2001, Partridge et al., 2004, Chen et al., 2009). It remained to be determined however, if a reduction in CD46 glycosylation was associated with the downregulation of cell surface CD46 observed upon TCR activation. Changes in glycosylation could, for example, alter the conformation of surface CD46 and enhance its sensitivity to proteolysis or internalisation. In order to determine whether a decrease in O- or N-glycans could enhance CD46 downregulation, O- and N-glycosylation was inhibited using broad-spectrum inhibitors. O-glycosylation was inhibited with Benzyl-α-GalNAc (5 mM) and N-glycosylation was inhibited with tunicamycin (15 μg/ml). T cells were incubated overnight with the O- or N-glycosylation inhibitors or their respective controls, methanol and DMSO. The following day the T cells were left unstimulated or stimulated with αCD3, αCD3/CD46 or αCD46 alone. After 24 hrs stimulation CD46 cell surface expression was determined. Despite having no effect on unstimulated, αCD3 or αCD46 stimulated cells, treatment with the O-glycosylation inhibitor, Benzyl-α-GalNAc, attenuated the downregulation of CD46 upon αCD3/CD46 stimulation (*figure 5.5A*). In contrast, the N-glycosylation inhibitor, tunicamycin, induced a reduction in CD46 expression upon αCD3/CD46 and αCD46 stimulation, with the greatest reduction observed upon αCD3/CD46 stimulation (*figure 5.5B(i)*). This reduction in CD46 expression was also observed after intracellular staining for CD46 (*figure 5.5B(ii)*) suggesting that the decrease was not due to internalisation (excluding degradation). These data indicate that inhibition of N-glycosylation promotes CD46 downregulation, and in contrast inhibition of O-glycosylation attenuates CD46 downregulation upon CD46 costimulation. Therefore it is possible that TCR induced N-deglycosylation could play a role in promoting CD46 downregulation.

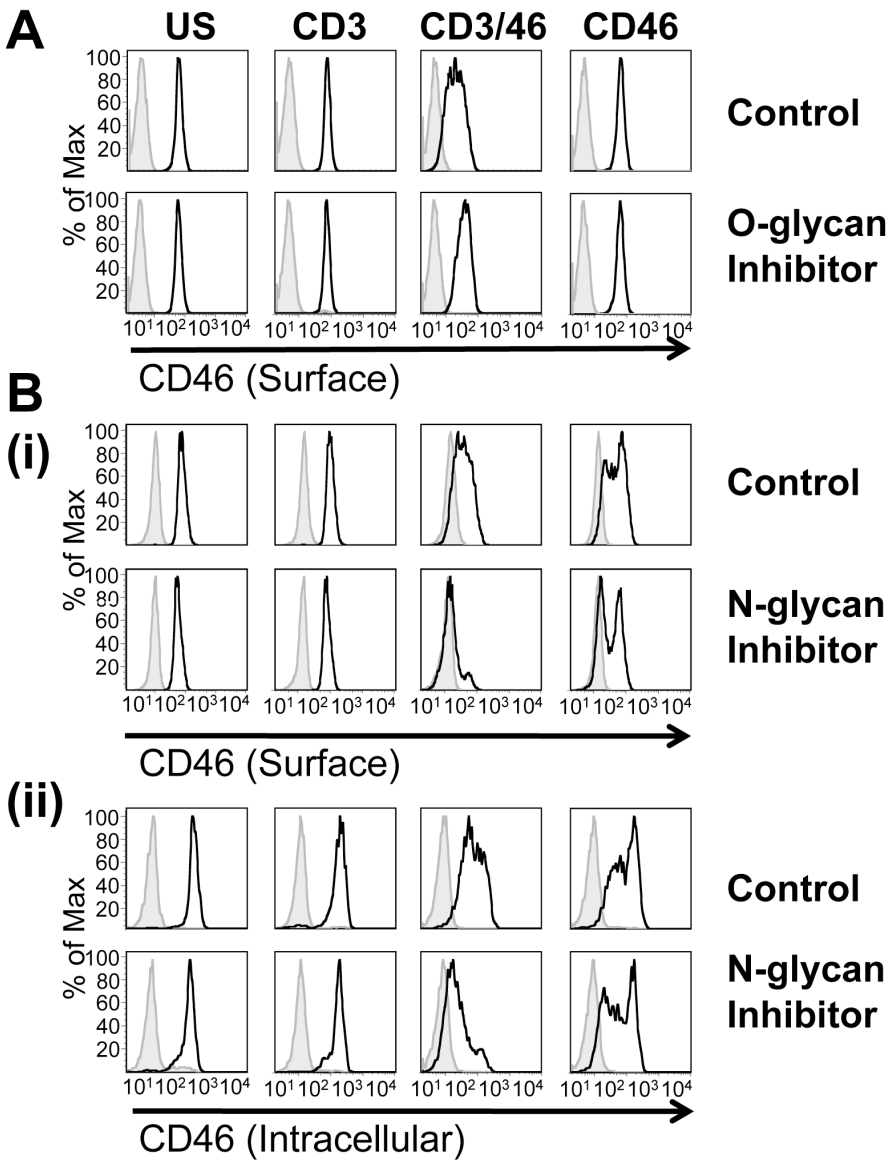


***Figure 5.5: Broad-spectrum inhibition of O- and N-glycosylation have opposing effects on CD46 expression****. CD4$^+$ T cells were incubated overnight with Benzyl-α-GalNAc (O-glycan inhibitor)(5 mM) or tunicamycin (15 μg/ml)(N-glycan inhibitor) and their respective controls, methanol or DMSO. The next day, cells were left unstimulated (US) or activated for 24 hrs with αCD3 (CD3), αCD3/CD46 (CD3/46) or αCD46 (CD46) and CD46 expression was determined by flow cytometry.* ***(A)*** *Surface CD46 expression of T cells incubated with the O-glycan inhibitor (representative of 2 independent experiments).* ***(B)*** *CD46 expression of cells treated with N-glycan inhibitor* ***(i)*** *Surface CD46 expression (representative of 9 independent experiments) and* ***(ii)*** *Intracellular CD46 expression (representative of 4 independent experiments).*

### 5.4.5 The effect of the O- and N-glycosylation inhibitors on CD69 and cytoplasmic tail expression

*Chapter 4* demonstrated that proteolysis of CD46 can regulate T cell activation. Therefore, as inhibition of N- and O-glycosylation regulated surface expression of CD46 it was hypothesised that N- and O-glycosylation may also affect T cell activation. To examine the state of activation of cells treated with the O- or N-glycosylation inhibitors, CD69 expression was determined after overnight activation. Inhibition of O-glycosylation appeared not to affect CD69 expression whereas the inhibition of N-glycosylation decreased CD69 expression. Of note, under all conditions increased CD69 expression correlated with the downregulation of CD46 (*figure 5.6A*). These data support the conclusion made in *Chapter 4* that downregulation of CD46 is important for T cell activation.

It was demonstrated in *Chapter 4* that the downregulation of CD46 and the proteolytic cleavage of its intracellular tail Cyt1, facilitated T cell activation. As inhibition of N-glycosylation promoted the downregulation of CD46 it was also examined if cell surface downregulation correlated with alterations in the expression of the cytoplasmic tails. The expression levels of both cytoplasmic isoforms were determined by flow cytometry after overnight activation in the presence of the N-glycosylation inhibitor, tunicamycin. Upon αCD3/CD46 activation there was an increased downregulation of both tails, although, the effect was stronger for Cyt1 than Cyt2 (*figure 5.6B*). Overall these results indicate that a lack of N-glycans on CD46 increases its cell surface downregulation, which is mirrored in the downregulation of its cytoplasmic tails, particularly Cyt1.

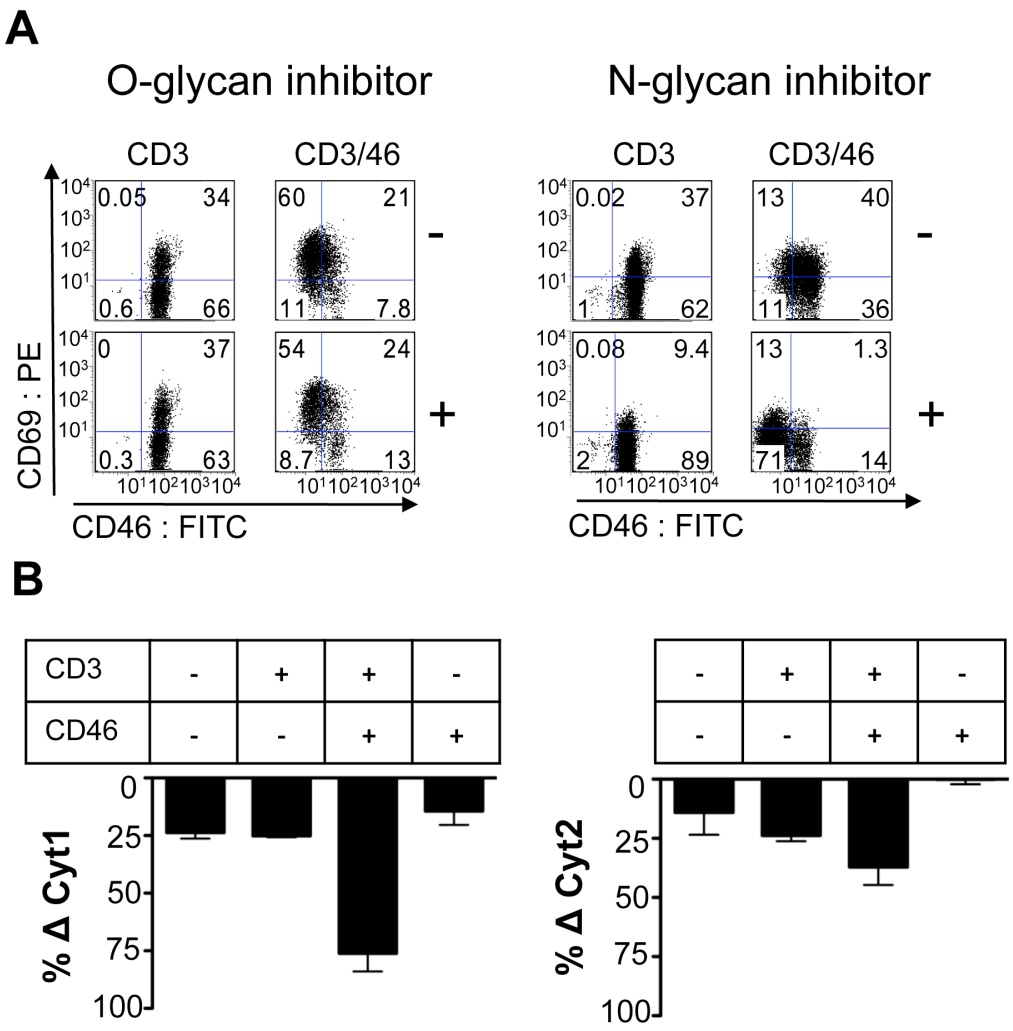


***Figure 5.6: The effect of the O- and N- glycosylation inhibitors on CD69 and cytoplasmic tail expression***. ***(A)*** *T cells were incubated overnight with the O-glycosylation inhibitor (Benzyl-α-GalNAc) or the N-glycosylation inhibitor (tunicamycin), alongside their respective controls, and activated as indicated the following day. After overnight stimulation CD69 expression was determined by flow cytometry (left panel is representative of 4 independent experiments, right panel is representative of 2 independent experiments).* ***(B)*** *Cyt1 and Cyt2 expression was determined by flow cytometry. The percentage change (%Δ) in cyt1 and cyt2 downregulation in the presence of the N-glycosylation inhibitor compared to controls was calculated (n=2).*

### 5.4.6 The effect of the O- and N-glycosylation inhibitors on cytokine secretion upon CD46 costimulation

TCR activation synergises with CD46 to promote its cleavage *(Figure 5.2)*. and also induced the expression of a lower glycosylated variant of CD46 (*figure 5.4*). Glycosylation changes have been shown previously to alter the surface expression of other receptors (Tifft et al., 1992, Demetriou et al., 2001, Partridge et al., 2004, Lau et al., 2007, Chen et al., 2009, Grigorian et al., 2012). Thus, by decreasing the $M_w$ of CD46, TCR activation may regulate CD46 cleavage and also its function. Therefore, as the O- and N-glycosylation inhibitors had contrasting roles on CD46 expression, it was also determined if the inhibitors altered CD46 induced cytokine secretion. T cells were treated with the O-glycosylation inhibitor (1 mM and 3 mM) and were cultured for 5 days before collecting the supernatants to analyse IFNγ and IL-10 secretion by ELISA. The O-glycosylation inhibitor increased IFNγ secretion, in a dose dependent manner following both αCD3 and αCD3/CD46 stimulation. Upon calculation of the IL-10:IFNγ ratio there was a dose dependent decrease in the ratio upon both αCD3 and αCD3/CD46 stimulation compared to the controls (*figure 5.7A*). These results indicate that inhibition of O-glycosylation attenuates CD46 cell surface downregulation and that this correlates with a decreased ratio of IL-10:IFNγ.

Neither IFNγ nor IL-10 could be detected by ELISA in the presence of the N-glycosylation inhibitor (*data not shown*) because the inhibitor had significantly reduced the activation state of the T cells. In attempt to detect changes in cytokine secretion, an IFNγ and IL-10 secretion assay was used to detect cytokine secretion after overnight stimulation. A few cells secreted IFNγ but there was almost no detection of IL-10 (*data not shown*). This may be due to the sensitivity of the IL-10 secretion assay or that IL-10 is secreted at a later timepoint as previously described (Cardone et al., 2010). Although, there was minimal IFNγ secretion, only the cells that had downregulated CD46 were positive for IFN-γ *(figure 5.7B)*. These data indicate that N-glycosylation is crucial to T cell activation. However, these results also suggest the T cells that downregulate

CD46 are more activated than those that do not as these were the only cells that were positive for cytokine secretion, emphasising the importance of CD46 downregulation during CD46 costimulation.

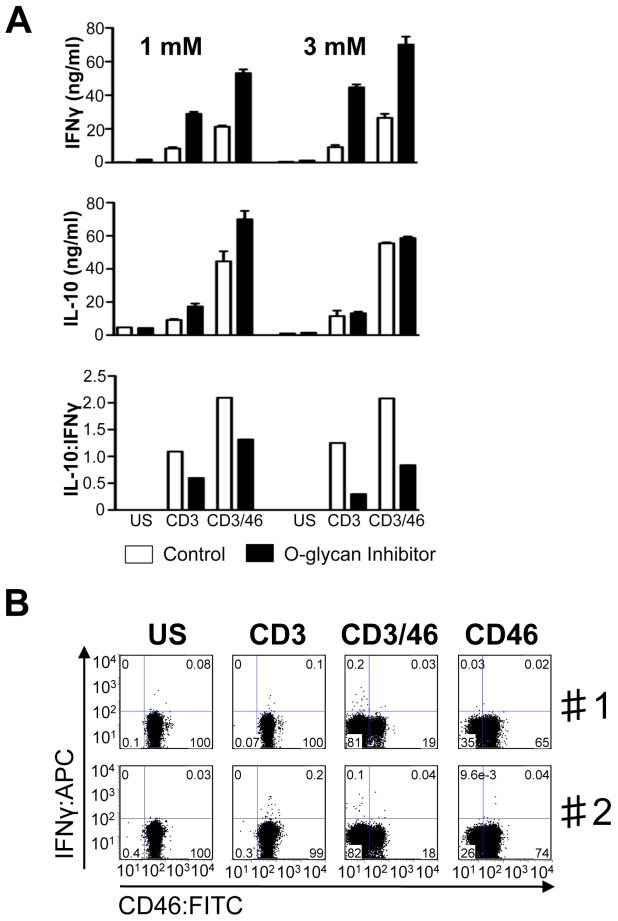


***Figure 5.7: The effect of the O- and N- glycosylation inhibitors on cytokine secretion upon CD46 costimulation**. **(A)** CD4⁺ T cells were incubated overnight with the O-glycosylation inhibitor (1 mM or 3 mM), or methanol as a control, then stimulated as indicated for 5 days. The concentration of IFNγ and IL-10 were quantified by ELISA. (n=1) **(B)** CD4⁺ T cells were incubated overnight with an N-glycosylation inhibitor and stimulated overnight with αCD3 (CD3) or αCD3/CD46 (CD3/46). IFNγ secretion was detected using the Miltenyi secretion assay (n=3), two independent experiments are represented.*

### 5.4.7 Specific inhibition of CD46 glycosylation differently affects CD46 cell surface expression in CHO cells

Broad-spectrum inhibition of either N- or O-glycosylation strongly affected CD46 cell surface expression. However, broad-spectrum inhibitors may also affect the glycosylation of other proteins involved in regulating T cell activation that may have indirect effects on CD46 surface expression. As CD46 surface expression is inherently linked with regulating its function, ascertaining which glycans affect CD46 cell surface expression would indicate a potential role of the glycans in regulating CD46 function during T cell activation. Alterations in CD46's glycosylation may possibly explain defective IL-10 secretion in patients with MS. Therefore mutant CD46 constructs were utilised to determine the specific effect of O- and N-glycosylation of CD46 on its cell surface expression. These constructs utilised the BC1 CD46 isoform as a template and were kindly provided by the Atkinson lab (Washington University, St. Louis, MO) (Maisner et al., 1996, Liszewski et al., 1998). CD46 contains multiple O-glycosylation sites and 3 N-glycosylation sites (*figure 5.1*). Specific mutations at these sites inhibited either N-glycosylation or O-glycosylation and therefore helped determine if N- or O-glycosylation of CD46 affected CD46 surface expression. As the STP region contains numerous O-glycosylation sites a single mutant with the STP region deleted (ΔSTP) was used to assess the effect of O-glycosylation. Specific mutations of asparagine (N) to glutamine (Q) at each of the three N-glycosylation sites, in the SCR1, SCR2 and SCR4 regions, were performed, thereby inhibiting specific N-glycosylation. These CD46 mutants were called NQ1, NQ2 and NQ4 respectively. A wild type BC1 CD46 isoform was used as a positive control. These constructs were expressed in CHO cells lines that did not express endogenous CD46 but had stable expression of each one of the mutants listed in *table 5.1*. A CHO cell line also expressed an unreadable reversed CD46 cDNA (BC1 (-)) and was utilised as a negative control (CHO cell lines were also provided by the Atkinson lab).

***Table 5.1: Control and mutant CD46 DNA constructs.***

| DNA Plasmid | Mutation |
|---|---|
| BC1 | Wild Type |
| ΔSTP | Deletion of STP region |
| NQ1 | SCR1 N➔Q |
| NQ2 | SCR2 N➔Q |
| NQ4 | SCR4 N➔Q |

Firstly, the $M_w$ of the BC1 mutants was determined by SDS-PAGE to confirm that the mutations in CD46 resulted in a decreased $M_w$ that reflected the absence of their specific glycans. The mobility of the wild-type BC1 was similar to that reported previously (~67 kDa) (Maisner et al., 1996, Liszewski et al., 1998). ΔSTP had the lowest $M_w$ (~55 kDa as it lacks numerous O-glycans) and all three of the N- mutants had a decreased $M_w$, consistent with the loss of one glycan, (~62 kDa) (*figure 5.8A*). Therefore, the mutant CD46 CHO cells lines expressed lower $M_w$ CD46 isoforms that reflected the inhibition of glycosylation at either a specific N-glycosylation site or the STP O-glycosylation region.

The specific effects of the mutant CD46 variants on surface downregulation were investigated. As CHO cells do not express endogenous CD46, the effects of the glycan mutants could be easily determined. CHO cells expressing the different CD46 mutants were plated with either IgG1 or αCD46, and CD46 expression was observed by flow cytometry after 24 hrs of stimulation. Although, variable levels of CD46 expression were observed for each cell line, CD46 ligation induced a downregulation of CD46 expression in all conditions. However, the strongest downregulation was observed in the BC1, NQ1, NQ2 and NQ4 CHO cells. In contrast, ΔSTP attenuated CD46 downregulation (*figure 5.8B*) suggesting that O-glycans were important for CD46 downregulation. Therefore, in CHO cells the absence of different glycans subsets can have opposing roles on CD46 downregulation.

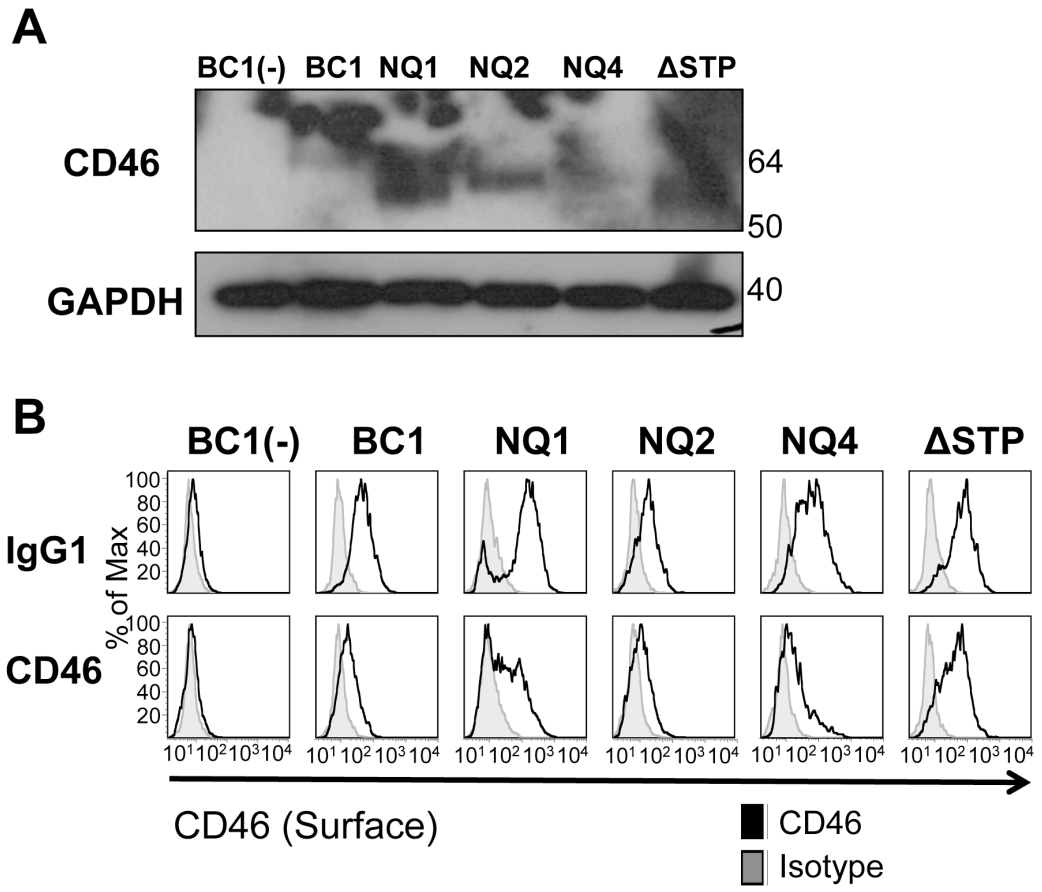


***Figure 5.8: Specific inhibition of CD46 glycosylation differently affects CD46 expression in CHO cells**. **(A)** The $M_w$ of CD46 in CHO cells expressing no CD46 (BC1 (-)), the BC1 isoform (BC1), or CD46 BC1 mutants with specific point mutations in SCR1 (NQ1), SCR2 (NQ2), SCR4 (NQ4) or an STP deletion (ΔSTP) were determined by SDS-PAGE (representative of 2 independent experiments). GAPDH was used as a loading control. **(B)** CHO cells expressing the mutants described in (A) were stimulated with plate bound IgG1 or αCD46 (CD46) for 24 hrs. After overnight stimulation CD46 expression was analysed by flow cytometry (representative of 3 independent experiments).*

### 5.4.8 Specific inhibition of CD46 O-glycosylation attenuates CD46 cell surface downregulation in T cells

The ΔSTP mutant cell line attenuated CD46 downregulation in CHO cells. These results correlate with *figure 5.5* which demonstrate that inhibiting O-glycosylation in T cells attenuated CD46 downregulation. In order to determine if the specific inhibition of CD46 O-glycosylation also attenuated CD46 downregulation in human T cells, T cells were transfected with the ΔSTP plasmid, wild-type BC1 plasmid or a control vector only (CVO) plasmid, as a negative control. Then the cells were left unstimulated or activated with αCD3, αCD3/CD46 or αCD46. CD46 expression was analysed by flow cytometry after overnight stimulation. Upon CD46 costimulation there is a strong downregulation of surface CD46 in all conditions. However, upon transfection with the ΔSTP plasmids there was a slight increase in CD46 expression compared to cells transfected with the BC1 or CVO plasmids. This increase was more pronounced in the absence of TCR stimulation. However, no increase in CD46 expression in the ΔSTP transfected cells was observed when the cells were left unstimulated or activated with CD3 alone (*figure 5.9A*). These data suggest that ΔSTP CD46 attenuates CD46 downregulation in T cells.

In *Chapter 3*, it was demonstrated that CD46 downregulation occurred as early as 5 hrs post ligation and was most strongly downregulated after overnight stimulation. As only a small effect of the ΔSTP plasmid was observed after overnight CD46 costimulation it was hypothesised that overnight stimulation may have been too late to observe a significant effect of the ΔSTP plasmid. Therefore, T cells were transfected with the ΔSTP plasmid and were stimulated for a period of 5 hrs. Increasing doses of ΔSTP (1 μg and 2 μg) were also used to maximise the transfection efficiency potential. Upon CD46 costimulation, a dose dependent increase in CD46 expression was observed compared to the control (*figure 5.9B*). These data confirm that ΔSTP transfected T cells have increased expression of CD46 upon CD46 costimulation.

CD46 expression was also assessed by SDS-PAGE in an effort to distinguish mutant CD46 expression from endogenous CD46 and assess if increased CD46 expression in the ΔSTP transfected cells represented the mutant ΔSTP CD46 or endogenous CD46. Detection of CD46 by SDS-PAGE demonstrated increased CD46 expression in ΔSTP transfected cells compared to controls. This increase in CD46 expression may be due to a lower $M_w$ band that reflected the size of the ΔSTP mutant, however this is difficult to confirm due to variations in protein concentration on the blot. (*figure 5.9C*). Overall, these data indicate that the ΔSTP mutant CD46 attenuates CD46 downregulation in human T cells. As the STP region is rich in O-glycans, these results suggest that O-glycosylation positively regulates CD46 downregulation.

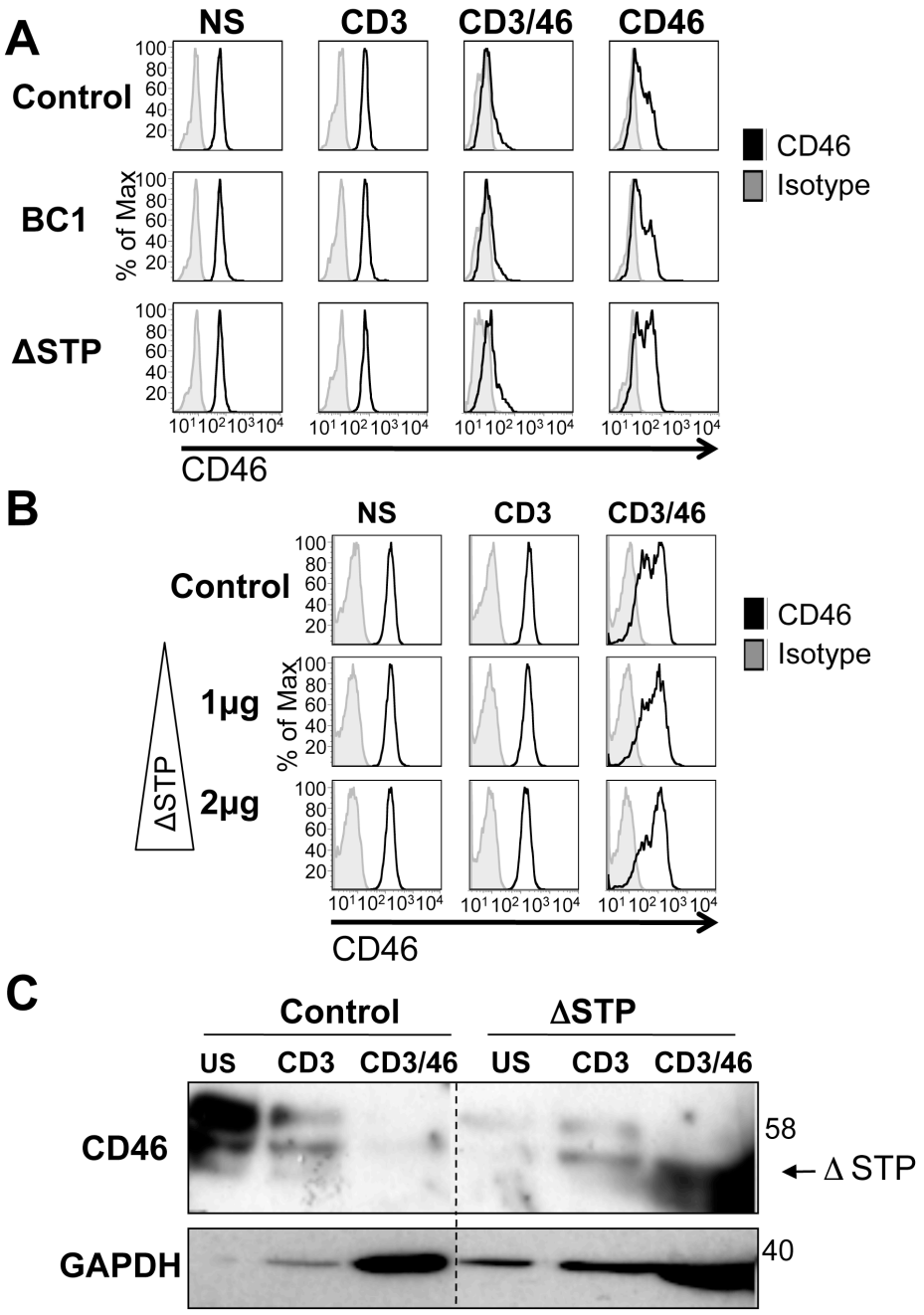


***Figure 5.9: In CD4$^+$ T cells, over expression of CD46, lacking the STP domain (ΔSTP) attenuates CD46 downregulation at the surface. (A)*** *T cells were transfected with 1 μg of the control vector only (CVO) as a negative control (control), wild-type BC1 or the ΔSTP mutant. Cells were left unstimulated or stimulated with αCD3 (CD3), αCD3/CD46 (CD3/46) or αCD46 (CD46) alone. CD46 expression was determined by flow cytometry after overnight stimulation (representative of 5 independent experiments).* ***(B)*** *T cells were transfected with 1 μg or 2 μg of the ΔSTP mutant cDNA and stimulated as indicated. CD46 expression was determined by flow cytometry after 5 hrs of stimulation (n=1).* ***(C)*** *Cells were transfected and stimulated as in (B). After 5 days stimulation, CD46 expression was determined by SDS-PAGE (n=1).*

### 5.4.9 Transfection of T cells with CD46 glycosylation mutants and its effect on CD46 surface expression and cytokine secretion

TCR activation promoted the expression of lower glycosylated forms of CD46 and its surface downregulation. Inhibition of N-glycosylation in T cells using the inhibitor tunicamycin suggests that the absence of N-glycan(s) promotes the downregulation of surface CD46. In contrast the presence of O-glycosylation appears to promote downregulation. These data suggest that reduced levels of N-glycans on CD46 could promote CD46 downregulation. The roles of N-glycans in regulating CD46 expression were therefore examined. T cells were transfected with the N-glycan mutants, NQ1, NQ2 and NQ4 alongside the wild-type BC1, the STP mutant and the negative CVO control. The cells were left unstimulated or activated with αCD3, αCD3/CD46 or αCD46 and CD46 cell surface expression was determined after overnight stimulation. No significant effects were observed in unstimulated or αCD3 stimulated cells (*data not shown*). Upon CD46 costimulation, both NQ1 and NQ4 had similar levels of CD46 expression as BC1. However, NQ2 showed an increase in CD46 expression (*figure 5.10A and figure 5.10B*). Of, note there was a large standard deviation in CD46 expression for the STP mutant, this is likely due to variations in transfection efficiency or the fact that CD46 expression was determined after overnight stimulation and not after 5 hours activation, which may be a more sensitive timepoint. Therefore, these results suggest that NQ2 could attenuate CD46 downregulation, and suggests that the N-glycan at the SCR2 domain may aid CD46 downregulation. The absence of glycans at SCR1 and SCR4 appear to play no significant role or may in fact reflect glycans that are removed upon TCR activation.

Downregulation of cell surface CD46, particularly the Cyt1 isoform, as a result of proteolysis plays an important role in regulating CD46 activation. As both the N-glycans and O-glycans appear to regulate cell surface CD46 expression, their effect on CD46 function was also assessed. T cells were transfected with the mutant plasmids as described above and were activated with αCD3/CD46. After 5

days stimulation, the concentration of IL-10 and IFNγ was determined by an IL-10 or IFNγ specific ELISA assay, respectively. Of note, in some conditions there was a large standard error mean which is likely due to differences in individual donor responses and may also be the result of differences in transfection efficiency. Over expression of CD46 mutants inhibiting N-glycosylation generally show a trend of increased IL-10 and IFNγ secretion compared to BC1. This is in contrast to the ΔSTP mutant that tends to decrease IL-10 but not IFNγ (*figure 5.10B(i) and (ii)*). The ratio of IL-10:IFNγ was also determined (*figure 5.10B(iii)*). ΔSTP showed a decreasing trend in IL-10:IFNγ compared to BC1. The mutants with higher levels of CD46 surface expression, NQ2 and ΔSTP, also appeared to have slightly lower ratios of IL-10:IFNγ secretion and NQ1 and NQ4, which had the lowest CD46 expression of the mutants had the highest ratio of IL-10:IFNγ secretion (*figure 5.10*). These data suggest that CD46 glycans could regulate CD46 surface expression and that this may be associated with the phenotype of cytokine secretion. Alterations in proliferation were also determined by [$^3$H] thymidine incorporation at day 3. The N-glycan mutants NQ1 and NQ2 showed a trend of increased proliferation compared to BC1, however the O-glycan mutant ΔSTP inhibited proliferation (*figure 5.10B(iv)*). Therefore, different glycosylation states may also affect T cell proliferation. Most notably, expression of ΔSTP CD46 that was shown to attenuate CD46 downregulation in some donors also attenuated T cell proliferation. The variation of results may reflect differences in transfection efficiency. Therefore, the glycosylation of CD46 in T cells affects surface expression of CD46 and this may correlate with its function. Further experiments examining the role of CD46 glycosylation in regulating cytokine secretion are required to confirm a direct link between CD46 glycans and its functions as a T cell receptor

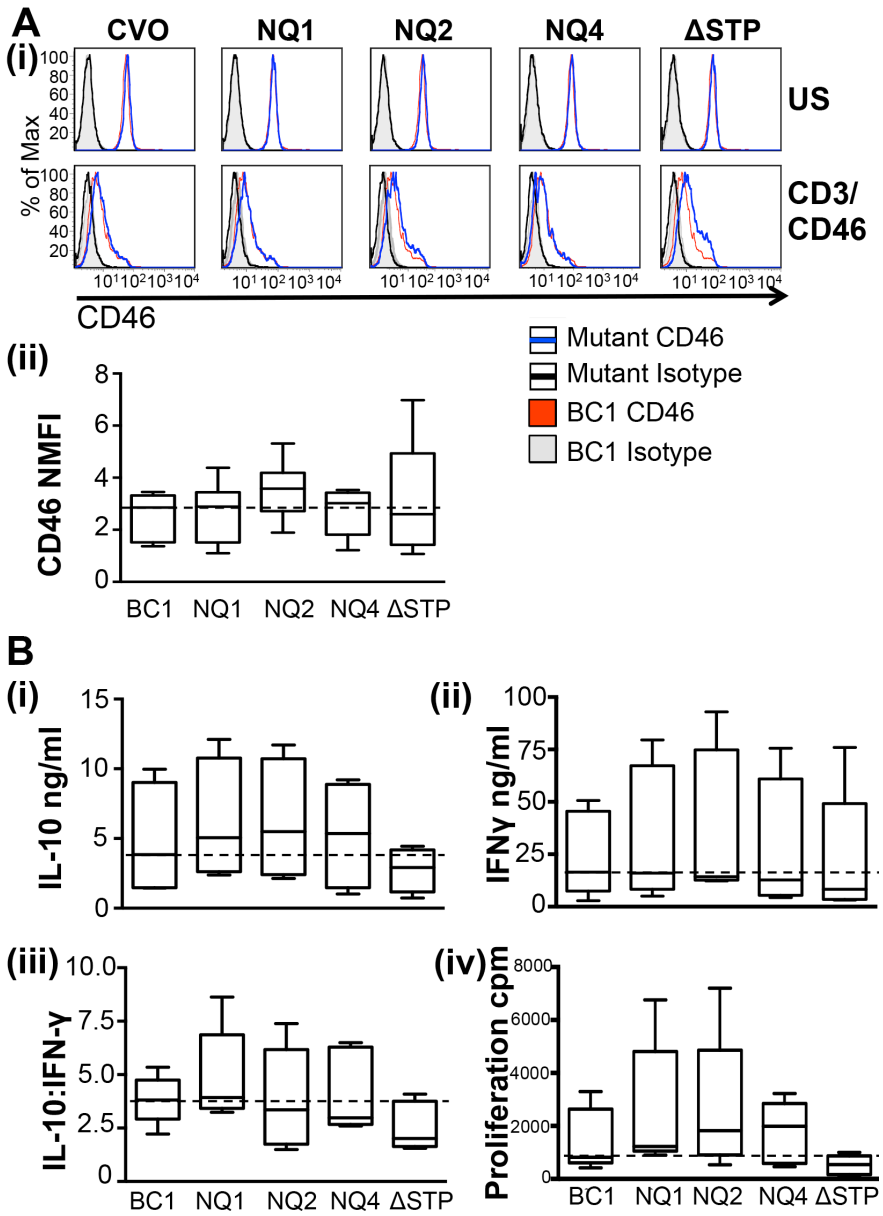


***Figure 5.10: Transfection of T cells with CD46 glycosylation mutants and their effect on CD46 surface expression and cytokine secretion****. T cells were transfected with an empty plasmid (CVO), the BC1 isoform (BC1) or a mutant BC1 isoform. Then, T cells were left unstimulated or stimulated with αCD3/CD46.* ***(A)*** *CD46 expression was determined by flow cytometry after overnight stimulation.* ***(i)*** *CD46 expression (blue) overlaid with the BC1 control (tinted grey)* ***(ii)*** *Mean CD46 surface expression upon αCD3/CD46 stimulation (n=5).* ***(B)*** *After 5 days αCD3/CD46 stimulation, the concentration of IL-10 and IFNγ were assessed by ELISA* ***(i and ii)*** *and the ratio of IL-10:IFNγ was calculated* ***(iii)****. Proliferation was determined after 3 days stimulation using thymidine incorporation* ***(iv)****, n=5.*

## 5.5 Discussion

***TCR induced changes in CD46 proteolysis and glycosylation***

TCR activation regulates CD46 surface expression by promoting its downregulation and proteolytic release of sCD46 (*figure 5.2*). TCR induced shedding has been previously described for the ITIM receptor CD31 (Fornasa et al., 2010). We have shown previously that cleavage of Cyt1 facilitates T cell activation (*Chapter 4*). TCR activation also induces a dose dependent decrease in the $M_w$ of CD46 that is also observed upon CD46 and CD28 costimulation, but not upon CD46 ligation alone demonstrating that TCR engagement induces a decrease in CD46 $M_w$ (*figure 5.3*). Indeed, preliminary data indicate that PMA/ionomycin treatment can also induce a decrease in the $M_w$ of CD46 (*data not shown*) suggesting that a decrease in CD46's $M_w$ may be inherently linked to T cell activation. The decrease observed in CD46's $M_w$ is the result of a reduction in CD46 glycosylation (*figure 5.4*). Three possible scenarios could explain the expression of a lower $M_w$ CD46; (i) TCR signalling induces the expression of newly synthesised CD46 with a decreased glycosylation state, (ii) there is a readily available pool of CD46 with a lower $M_w$ that is inaccessible to antibody detection in unstimulated cells but is accessible upon activation or (iii) deglycosylation of pre-existing CD46 occurs at the cell surface/within endosomes upon activation.

In the first scenario, expression of lower glycosylated forms of CD46 would also involve a rapid degradation of the higher glycosylated isoforms from the surface as the mobility of CD46 on SDS-PAGE is not observed as a blur but rather as a clean shift in $M_w$ (*figure 5.3*). This scenario is possible with respect to rapid new protein synthesis as mature protein expression has been reported to occur in 90 min in a monocyte cell line (Ballard et al., 1988). Nonetheless, such a rapid turnover seems unlikely considering the energy required and inefficiency of a complete change of protein expression within the cell. Scenario (ii) would involve the presence of a pre-existing pool of CD46 with a lower $M_w$ that is

recruited to the surface upon activation. However, as a lower $M_w$ CD46 is not observed in unstimulated cells this scenario would indicate that the pool was resistant to lysis buffer used in these experiments. CD46 may for example be located in multivesicular bodies and be rapidly sent to the surface for cleavage and downregulation. Indeed, it has long been known that cross-linking of receptors has the capacity to divert receptors away from cell surface recycling to the lysosomes for degradation (Mellman, 1996). However, lysis buffers similar to the one used in this study have been reported to readily lyse endosomal vesicles including multivesicular bodies (German and Howe, 2009) suggesting that this scenario is also unlikely. Scenario (iii) would involve deglycosylation of existing CD46 directly at the surface or intracellularly after endocytosis and seems more probable. Sialidase, which removes the sugar sialic acid from N- and O-glycans, is present at the surface membrane and in recycling endosomes (Zanchetti et al., 2007). Moreover, upon CD46 ligation, CD46 has been shown to translocate to lipid rafts (Ludford-Menting et al., 2011) where sialidases reside (Kalka et al., 2001). Furthermore, upon TCR ligation there is an upregulation of the sialidases, NEU1 and NEU3, in T cells (Wang et al., 2004, Nan et al., 2007). Therefore, it is possible that sialic acid from N- and/or O-glycans is removed upon T cell activation. However, deglycosylation is not limited to the removal of sialic acid. PNGase, that cleaves N-glycans, is also known to exist in mammalian cells (Suzuki et al., 1993) and can act to deglycosylate specific N-glycans (Suzuki et al., 1997). Removal of TCR and MHC class I N-glycans have also been reported to occur in the cytosol (Wiertz et al., 1996, Huppa and Ploegh, 1997) and in the endosomal/lysosomal pathway (Schmitt and Grand-Perret, 1999). Using the drug chloroquine in *Chapter 3*, it was determined that CD46 downregulation involved the endosomal pathway. Therefore, decreases in the $M_w$ of CD46 could occur after downregulation into the endosomal pathway. Further experiments would be required to determine which of these scenarios is correct.

The determination of the exact changes in CD46 glycosylation upon T cell activation would require complex mass spectrometry studies that were outside the

timeframe of this project. However, from these experiments it can be noted that the observed decrease in glycosylation upon activation is ~2 kDa (*figure 5.3*) and this decrease could potentially correspond to the removal of a N-glycan which is reported to be of similar $M_w$ (Ballard et al., 1988) or trimming of decorating sugars that are attached to N- or O-glycans, such as sialic acid. Importantly, lower levels of both N- and O-glycosylation have been previously implicated in the decrease of cell surface protein expression due to endocytosis and proteolytic cleavage (Lau et al., 2007, Van den Steen et al., 1998, Rudd et al., 1999). In accordance with the literature increased cleavage of CD46 as a result of TCR stimulation is demonstrated in *figure 5.2*. These results suggest that TCR induced CD46 cleavage and deglycosylation are inherently linked. A potential model that encompasses TCR induced deglycosylation and cleavage is depicted in *figure 5.11*. In this model, TCR activation (1) decreases glycosylation of CD46 either through deglycosylation or the expression of newly recruited CD46 with a lower level of glycosylation (2). Lower $M_w$ CD46 is more susceptible to cleavage and results in the release of the sCD46 domain and ICDs (3).

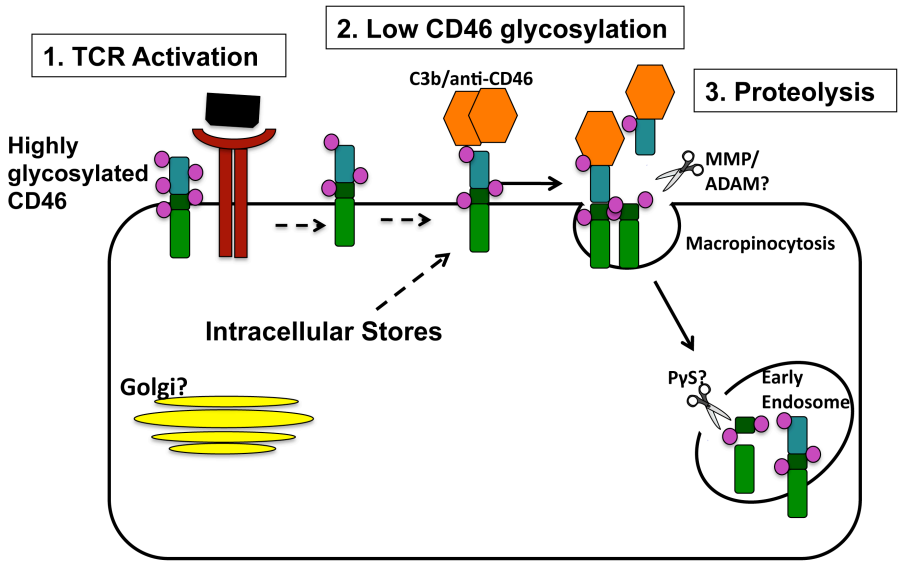


***Figure 5.11: Proposed model for CD46 processing and signalling upon T cell activation.***

***The role of glycosylation in CD46 downregulation***

The specific role of both N- and O-glycans in CD46 downregulation and function was examined using N- and O- glycosylation inhibitors and then by expressing mutant CD46 in CHO cells and T cells. These CD46 variants inhibited specific N-glycan attachment to CD46 using point mutations in the N-glycosylation sites or inhibited O-glycosylation by removing the STP region.

*O-glycans*

Both the inhibition of O-glycosylation (*figure 5.5*) and the expression of ΔSTP CD46 in CHO and T cells attenuated CD46 downregulation (*figure 5.8 and figure 5.9*). Indeed attenuation of CD46 downregulation was dose-dependent in T cells and was also observed upon CD46 ligation alone (*figure 5.9*). Therefore, O-glycosylation facilitates CD46 downregulation at the surface. Previous reports have shown that O-glycans provide protein rigidity or extension that protect against protease degradation (Reddy et al., 1989, Van den Steen et al., 1998) but in our system removal of the STP domain, and O-glycans, actually attenuated CD46 downregulation. Indeed, O-glycosylation can have varied effects depending on the receptor involved. CD44 is a transmembrane glycoprotein that binds hyaluronic acid (HA), which is involved in lymphocyte activation and the migration of cells (Gasbarri et al., 2003). Similar to CD46, CD44 also undergoes cell activation induced proteolytic cleavage. Importantly, PMA treatment induced proteolysis of a heavily O-glycosylated variant of CD44 in an O-glycan dependent fashion (Gasbarri et al., 2003). This report supports the role of CD46 O-glycans in facilitating TCR induced proteolysis.

*N-glycans*

Treatment of T cells with the N-glycosylation inhibitor, tunicamycin, suggests that N-deglycosylation of CD46 promotes its downregulation. Although, inhibition of N-glycosylation induced a slight decrease in CD46 in all conditions, there was a stronger decrease upon CD46 costimulation. The small decrease

observed is likely due to decreased protein stability and subsequent degradation, as previously reported (Maisner and Herrler, 1995). CD46 downregulation occurred at the surface and intracellularly after 24 hrs indicating that downregulation does not result in accumulation of CD46 intracellularly and may be the result of increased proteolytic cleavage (*figure 5.5B*). This does not however, exclude the possibility that CD46 is degraded upon internalization. In an attempt to examine this possibility, CD46 surface expression was determined at the surface and intracellularly at an early timepoint of 5 hrs. However no effect of tunicamycin was observed at this timepoint upon CD46 co-stimulation (*data not shown*). This may be due to insufficient time for new protein synthesis lacking N-glycans and/or the time taken for these proteins to repopulate the surface. Indeed, this also argues against the scenario *(i)* of deglycosylation previously discussed, where upon TCR activation there is rapid *de novo* protein synthesis and a complete protein turnover of lower glycosylated forms of CD46.

It is generally accepted that broad-spectrum inhibitors can have varying effects on protein expression and function. Therefore, in order to eliminate unspecific effects of the inhibitors in the analysis of CD46 N-glycosylation, CD46 mutants were utilised. Specifically, site-specific mutations were carried out at the 3 N-glycosylation sites of the BC1 isoform. These mutants, NQ1, NQ2 and NQ4 were stably expressed in CHO cells (Maisner et al., 1996, Liszewski et al., 1998). Upon CD46 ligation, all of the N-glycan mutants induced downregulation of cell surface CD46 (*figure 5.8*). After transfection of these mutants into human T cells, BC1, NQ1 and NQ4 transfected cells showed the strongest levels of downregulation (*figure 5.10*). Of note, transfection of T cells with the NQ2 mutant induced a small increase in CD46 expression compared to controls. Attenuation of CD46 downregulation in the NQ2 mutants suggests that N-glycosylation at SCR2 promotes CD46 downregulation. Interestingly, the SCR2 N-glycan is essential for measles virus infection (Maisner et al., 1996). Measles virus induces downregulation and internalisation of CD46 (Naniche et al., 1993) and can activate T cells via CD46 (Marie et al., 2002). Whether binding of measles virus induces shedding has not been addressed. However, binding of

measles virus to CD46 alters its conformation from bent to straight (Santiago et al., Persson et al., 2010) and N-glycosylation at SCR2 is believed to support the conformation required for measles virus ligation (Maisner et al., 1994, Maisner et al., 1996). This change in conformation may play a role in its downregulation and increase accessibility of proteases that cleave CD46.

As neither O-glycans nor the SCR2 N-glycan promote CD46 cleavage upon costimulation, the question of what glycans are removed/trimmed upon TCR ligation needs to be addressed. Neither the N-glycan on SCR1 or SCR4 had a considerable effect on CD46 expression compared to the BC1 control. However, it is worth highlighting that the expression of the BC1 wild type protein in T cells should, in theory, undergo deglycosylation similarly to endogenous CD46 upon activation. Therefore, if endogenous BC1 undergoes deglycosylation at SCR1 and/or SCR4 upon activation, CD46 expression levels observed would be similar to that of the mutants with a fixed deglycosylation state (i.e. the NQ1 and NQ4 mutants). Therefore, N-glycan mutants NQ1 and NQ4 may reflect native deglycosylation of the BC1 isoform upon T cell activation. In accordance with this hypothesis, treatment of T cells with the N-glycosylation inhibitor, tunicamycin, resulted in decreased CD46 expression (*figure 5.5*), suggesting that deglycosylation of N-glycans, namely N-glycans at SCR1 and/or SCR4, may play a role in promoting CD46 downregulation. It was shown in *figure 5.2* that the TCR synergises with CD46 to increase cleavage and deglycosylation. It is therefore proposed that the downregulation of cell surface CD46 observed upon N-glycosylation inhibition with tunicamycin and the expression of mutant N-glycan CD46 variants is at least partially the result of cleavage. Proteolytic cleavage of low-density lipoprotein (Kozarsky et al., 1988) and APP (Galbete et al., 2000) are also regulated by their glycosylation and suggest that glycosylation of CD46 could play a direct role in its proteolysis. Nonetheless, further experiments are required to confirm if decreases in CD46 glycosylation at the SCR1 and SCR4 domains are directly linked to increased proteolytic cleavage.

***Glycosylation and CD46 function***

*Chapter 3* demonstrated that the cleavage of CD46 is important for its function in activation. Therefore, given that changes in glycosylation have differential effects on CD46 expression it is highly suggestive of a role for CD46 glycans in CD46 function in T cell activation.

*The function of CD46's O-glycans*

The removal of the STP attenuated downregulation of CD46 in T cells (*figure 5.9 and figure 5.10*) and appeared to decrease T cell proliferation and the ratio of IL-10:IFNγ (*figure 5.10*). Although the removal of the STP region is a blunt tool for inhibiting O-glycosylation, broad-spectrum O-glycosylation inhibition suggests that O-glycosylation itself is required for CD46 downregulation (*figure 5.5*). Moreover, the O-glycosylation inhibitor also attenuated the IL-10:IFNγ ratio (*figure 5.7*). Thus, removal of O-glycans attenuate CD46 cell surface downregulation resulting in decreased T cell proliferation and IL-10:IFNγ secretion. Therefore CD46's O-glycans may be important for promoting CD46 induced regulatory T cells.

The mechanism of O-glycan support for CD46 downregulation and function remains to be determined. Using crystallography studies, it has been previously reported that STP splicing and O-glycosylation could affect the conformation of CD46 and the subsequent strength of ligand binding (Persson et al., 2010). Therefore, upon stimulation, CD46 O-glycans in the STP region may provide conformational support for ligand binding facilitating subsequent downregulation. The importance of O-glycans for CD46's function has been previously reported for complement inactivation and pathogen binding (Liszewski and Atkinson, 1996, Kurita et al., 1995, Liszewski et al., 1998). The splicing of the STP region is inherently linked to the level of CD46's O-glycosylation. CD46 isoforms that contain all 3 exons of the STP region, ABC have 18 O-glycosylation sites, whereas CD46 isoforms that consist of the C exon

only have 2 O-glycosylation sites (http://www.uniprot.org/uniprot/P15529, accessed 09/02/12). Cell and tissue specific expression of STP isoforms suggests a biological significance. For example, STPABC that is less commonly expressed but has increased expression in intestinal epithelial cell lines (Xing et al., 1994). *Cardone et al* have demonstrated that CD46 activation in epithelial cells promoted proliferation and wound repair upon bacterial infection (Cardone et al., 2011). It was demonstrated in *Chapter 4* that Cyt1 facilitates T cell activation, as measured by CD25 expression and proliferation. Therefore, increased O-glycosylation of CD46 in intestinal cells could also help promote CD46 cleavage and promote proliferation in the intestine. Higher O-glycosylated forms of CD46 (56-80 kDa) are also expressed in granulocytes conferring them with stronger co-factor I mediated cleavage of C3/C4 components (Seya et al., 1988, Kurita et al., 1995). Therefore O-glycans likely play various roles in CD46 function. Moreover, if O-glycans promote proteolysis of CD46 in T cells it is likely that they may also regulate CD46 processing in other cells types, emphasising the need for further investigation in this area.

*The function of CD46's N-glycans*

Despite several isoforms of CD46, it is worth taking note that N-glycosylation sites at SCR2 and SCR4 are conserved across humans, several old world monkeys and new world monkeys. Both humans and old world monkeys also retain the SCR1 glycan site (Hsu et al., 1997). This evolutionary conservation of N-glycan sites in CD46 emphasises their importance to its function. Upon CD46 costimulation, CD46 is cleaved at the surface by MMP/ADAM(s) and intracellularly by PγS, releasing functional ICD. *Chapter 3* proposes a model in which Cyt1 is cleaved early during T cell activation, facilitating T cell activation and cleavage of Cyt2 followed at a later time point and may promote T cell shutdown (*Chapter 4* and (Ni Choileain et al., 2011)). Indeed, upon the addition of the N-glycosylation inhibitor, CD46 surface downregulation is promoted and correlated with a strong downregulation of Cyt1 but not Cyt2 (*figure 5.6*). Although, tunicamycin suppresses normal T cell activation, it is worth noting that

the cells with strong CD46 downregulation increase CD69 expression and cytokine secretion compared to cells that retain CD46 expression (*figure 5.6 and figure 5.7*). This data supports the role of CD46 downregulation, specifically Cyt1, in promoting T cell activation.

In assessing N-glycan function using the mutant CD46 isoforms, all three N-glycan mutants showed a general trend for increased proliferation, IL-10 and IFNγ secretion compared to BC1. However, there are variations in the extent to which each mutant increases IL-10 and IFNγ secretion which gives rise to different ratios of IL-10:IFNγ (*figure 5.10*). Of note, there is a resemblance between the pattern of CD46 surface expression in the mutants and their IL-10:IFNγ secretion profile. Increased CD46 expression appears to inversely correlate with decreased IL-10:IFNγ (i.e. STP and NQ2). Conversely, stronger downregulation of surface CD46 expression in the mutant plasmids correlates with an increased IL-10:IFNγ ratio (i.e. NQ1 and NQ4) (*figure 5.10*). In summary, these data suggest that deglycosylation of CD46; at different glycosylation sites may alter its expression levels and its cytokine secretion profile. However, further experiments are required to be carried out to confirm these observations.

## 5.6 Conclusions

- TCR activation induces the expression of lower glycosylated CD46 and promotes its cleavage at the surface.
- The ΔSTP and NQ2 mutants attenuate CD46 surface downregulation.
- T cells transfected with the NQ1 and NQ4 mutants had a similar level of CD46 cell surface downregulation as wild-type BC1 transfected T cells.
- N-deglycosylation at different N-glycan sites of CD46 may affect the IL-10:IFNγ ratio secretion profile upon CD46 costimulation.
- O-deglycosylation and ΔSTP transfection of T cells shows a trend for a decreased IL-10:IFNγ ratio upon CD46 costimulation compared to control transfected T cells.

# Chapter 6 : CD46 expression in $CD4^+$ T cells from patients with Multiple Sclerosis

## 6.1 Introduction

As previously discussed, CD46 induced IL-10 secretion by T cells is dysfunctional in MS patients (Astier et al., 2006, Martinez-Forero et al., 2008, Ma et al., 2009). Moreover, CD46 dysfunction is not limited to MS and is also present in asthma (Xu et al., 2010, Tsai et al., 2012) and rheumatoid arthritis (Cardone et al., 2010) suggesting a more widespread role of CD46 in immune regulation. Understanding the mechanism of CD46 costimulation in healthy controls is key to understanding its dysregulation in MS patients and other pathologies.

In *Chapter 3*, regulated expression of surface CD46 and the Cyt1 and Cyt2 isoforms was described. Importantly, upon CD46 costimulation CD46 was proteolytically cleaved at the surface by MMP/ADAM(s) and intracellularly by PγS. The cleavage of Cyt1 and Cyt2 correlated with a time-dependent downregulation of expression. The cleavage of Cyt1 and possibly Cyt2 was important for their function in regulating T cell activation. (*Chapter 4*) (Ni Choileain et al., 2011). Moreover, it was also found that a decrease in CD46 glycosylation occurred upon T cell activation and could play a role in regulating CD46's expression and function (*Chapter 5*). These novel data have helped shed light on how CD46 expression and processing regulates cytokine secretion by T cells from healthy controls. Importantly, these new findings can now be used to investigate the pathways that are dysregulated in CD46 activated T cells from MS patients, which could explain why there is little or no IL-10 secretion in CD46 costimulated T cells from these patients (Astier et al., 2006).

In order to determine why CD46 function is dysregulated in T cells from relapsing-remitting MS (RRMS) patients, the expression levels of surface CD46 and intracellular Cyt1 and Cyt2 were first assessed. Herein, the dysregulated expression of CD46 by T cells from MS patients is reported. Specifically it is shown that upon CD46 costimulation there was no downregulation of Cyt1 or Cyt2 in MS patients, irrespective of interferon-β (IFNβ) treatment. Secondly, preliminary studies also suggest that T cells from RRMS patients have aberrant levels of CD46 glycosylation upon activation compared to healthy controls. Based on these results, it is hypothesised that dysregulated CD46 expression and/or glycosylation may disrupt CD46 processing, which is important for CD46-induced IL-10 secretion.

## 6.2 Aims

1. To determine CD46 cell surface expression by $CD4^+$ T cells from RRMS patients.
2. To examine the expression of the Cyt1 and Cyt2 isoforms in $CD4^+$ T cells from RRMS patients.
3. To assess the $M_w$ of CD46 in unstimulated and activated $CD4^+$ T cells from RRMS patients

## 6.3 Approach

In order to determine if dysregulated CD46 expression played a role in reducing IL-10 secretion in RRMS patients, the findings observed in *Chapter 3* and *Chapter 5* regarding CD46 expression levels and glycosylation during T cell activation were examined in a cohort of RRMS patients. These findings were compared to healthy controls in order to assess if there was defective expression or glycosylation of CD46 in patients.

Firstly, the expression levels of cell surface CD46 in $CD4^+$ T cells from RRMS patients were compared to controls. Similar to the experiments carried out using healthy control donors in *Chapter 3,* cell surface CD46 expression on $CD4^+$ T cells was determined throughout a five-day period. T cells were either left unstimulated or stimulated with αCD3, αCD3/CD46 or αCD3/CD28. Expression levels were then assessed at an early timepoint (day 1 or day 2) and a later timepoint (day 4 or day 5) and the results were compared with those obtained for healthy controls in *Chapter 3*. Due to limited numbers of patient donors, the MS cohort consisted of those who were both untreated and IFNβ treated at the time of donation. However, in an attempt to determine if treatment affected CD46 cell surface expression (despite low numbers), the patient cohort was also split into two groups (treated and untreated) and these results were compared to CD46 expression levels in healthy controls. In addition to cell surface CD46 expression, Cyt1 and Cyt2 expression were also assessed in RRMS patients at both the early and late timepoint. Expression levels of Cyt1 and Cyt2 were first compared between healthy donors and patients with MS (untreated and treated). Then, expression levels were compared between healthy donors, untreated patients and treated patients.

In *Chapter 5*, glycosylation was shown to play a potential role in regulating CD46's function. Therefore, preliminary experiments were also performed to explore the possibility that differences in glycosylation could be responsible for

the dysregulated IL-10 secretion in MS patients. Therefore the $M_w$ of CD46 upon activation was examined in T cells from a small group of MS patients.

# 6.4 Results

## 6.4.1 Cell surface CD46 expression is downregulated upon CD46 costimulation in $CD4^+$ T cells from patients with MS

As downregulation and cleavage of CD46 is important for its function in CD46 costimulated cells, cell surface CD46 downregulation was assessed in $CD4^+$ T cells isolated from MS patients. T cells were left unstimulated or activated with αCD3 or αCD3/CD46 and CD46 expression was determined by flow cytometry at either an early activation timepoint (after 1-2 days stimulation) or a late activation timepoint (after 4-5 days stimulation). These results were then compared to those described in *Chapter 3* for healthy controls, where CD46 expression was determined under comparable conditions. At both timepoints, upon CD46 costimulation, T cells from MS patients, downregulated CD46 cell surface expression compared to unstimulated and CD3 activated T cells. These findings were similar to that observed in healthy controls. However, at the later timepoint, αCD3 activated T cells from MS patients showed an increased CD46 expression compared to healthy controls (*figure 6.1*). Thus, despite the initial downregulation of CD46 at the earlier timepoints, T cells from MS patients exhibit increased expression of CD46 at the later timepoint in CD3 activated T cells. Overall this suggests that dysregulated CD46 expression may be present in T cells from MS patients and is investigated further below.

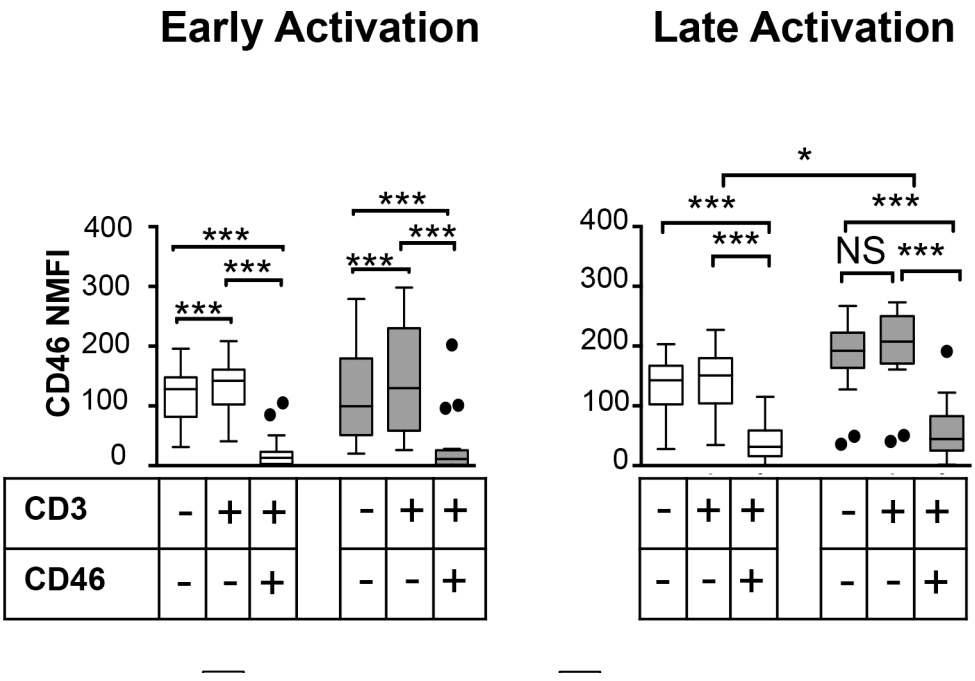


***Figure 6.1: CD46 is downregulated upon CD46 costimulation in CD4$^+$ T cells from MS patients.*** *T cells from MS patients were left unstimulated or activated in the presence of αCD3 alone or in the presence of αCD46. Cell surface CD46 expression was determined by flow cytometry at an early activation (1-2 days stimulation) or late activation (4-5 days stimulation) timepoint. Normalised mean fluorescence intensity (NMFI) was calculated. For comparison MS CD46 expression (grey) is presented alongside expression levels previously obtained for healthy controls (white) under similar conditions (Chapter 3). (**Left Panel**) CD46 expression during early activation (healthy controls (HC) n=25, MS patients n=21) (**Right Panel**). CD46 expression during late activation (HC n=18, MS patients n=15). A Friedman Test and Bonferroni-corrected Wilcoxon test was used for statistical analysis within the same donors, A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *** p≤0.0003 **p≤0.003, *p≤0.0167.*

### 6.4.2 CD46 expression upon CD28 costimulation in T cells from MS patients compared to healthy controls

Anti-CD3 activation of $CD4^+$ T cells from MS patients suggested that there was increased expression of cell surface CD46 surface compared to healthy controls (*figure 6.1*). It is possible that this dysregulated expression of CD46 could play a role in attenuating IL-10 secretion upon CD46 costimulation in MS patients. Dysregulated CD46 expression in activated T cells prior to CD46 ligation could also modify CD46 function. Thus, cell surface CD46 expression was also determined upon CD28 costimulation in order to elucidate if activated T cells from MS patients could have altered expression of CD46. $CD4^+$ T cells were left unstimulated or activated with αCD3 or αCD3/CD28. CD46 expression was then determined by flow cytometry at either an early activation timepoint or a late activation timepoint. Upon CD28 costimulation, T cells from MS patients and healthy controls appeared to have similar levels of CD46 expression at the early timepoint. However, upon CD28 costimulation at the later timepoint, MS patients had increased trend of CD46 compared to T cells from healthy controls (*figure 6.2*). Therefore, CD46 cell surface expression may be dysregulated in CD28 costimulated T cells from MS patients and could participate in altered CD46 responses upon T cell activation.

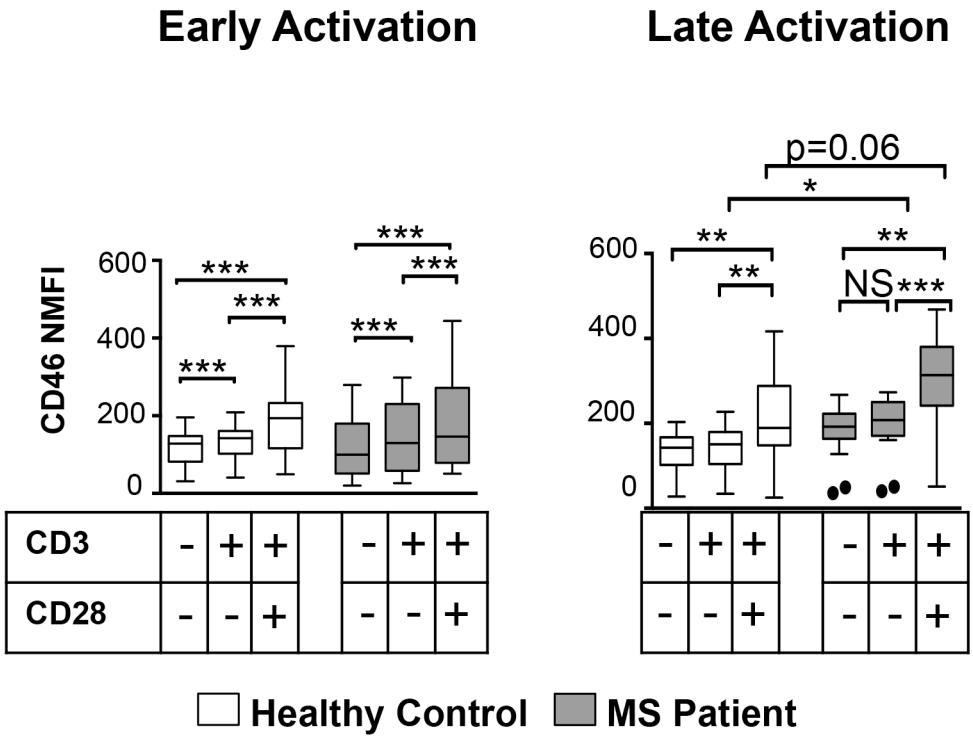


***Figure 6.2: CD46 expression upon CD28 costimulation in T cells from MS patients compared to healthy controls.*** *T cells from MS patients were left unstimulated or activated in the presence of αCD3 alone or in the presence of αCD28. Surface CD46 expression was determined by flow cytometry during early activation (1-2 days stimulation) or late activation (4-5 days stimulation) and the normalised mean fluorescence intensity (NMFI) was calculated. For comparison, MS CD46 expression (grey) is presented alongside expression levels previously obtained for healthy controls (white) under similar conditions (Chapter 3). (**Left Panel**) CD46 expression during early activation, (HC n=25, MS patients n=21). (**Right Panel**) CD46 expression during late activation, (HC n=18, MS patients n=16). A Friedman Test and Bonferroni-corrected Wilcoxon test was used for statistical analysis within the same donors, A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *** p≤0.0003 **p≤0.003, *p≤0.0167.*

### 6.4.3 CD46 expression levels in treated and untreated MS patients

In order to determine any potential effect of IFNβ treatment on CD46 expression in T cells from MS patients, CD46 expression levels in untreated and treated groups were compared separately to the expression levels previously observed for healthy controls. At the time of blood donation the treated group were receiving IFNβ treatment and the untreated group were not receiving any disease modifying drugs. At the early activation timepoint untreated patients tend to have lower levels of CD46 expression in all conditions compared to healthy controls, although this observation was not significant. In contrast, patients receiving IFNβ treatment appear to have higher expression levels compared to healthy controls (*figure 6.3*). At the later timepoint, $CD4^+$ T cells from MS patients, irrespective of treatment, appear to have increased levels of CD46 upon CD28 costimulation, compared to T cells from healthy controls. (*figure 6.3*). As the numbers within untreated and treated groups are low, these data are preliminary only. Despite this, however, these data suggest that CD46 expression could inherently defective in patients with MS and that IFNβ acts to modulate its expression.

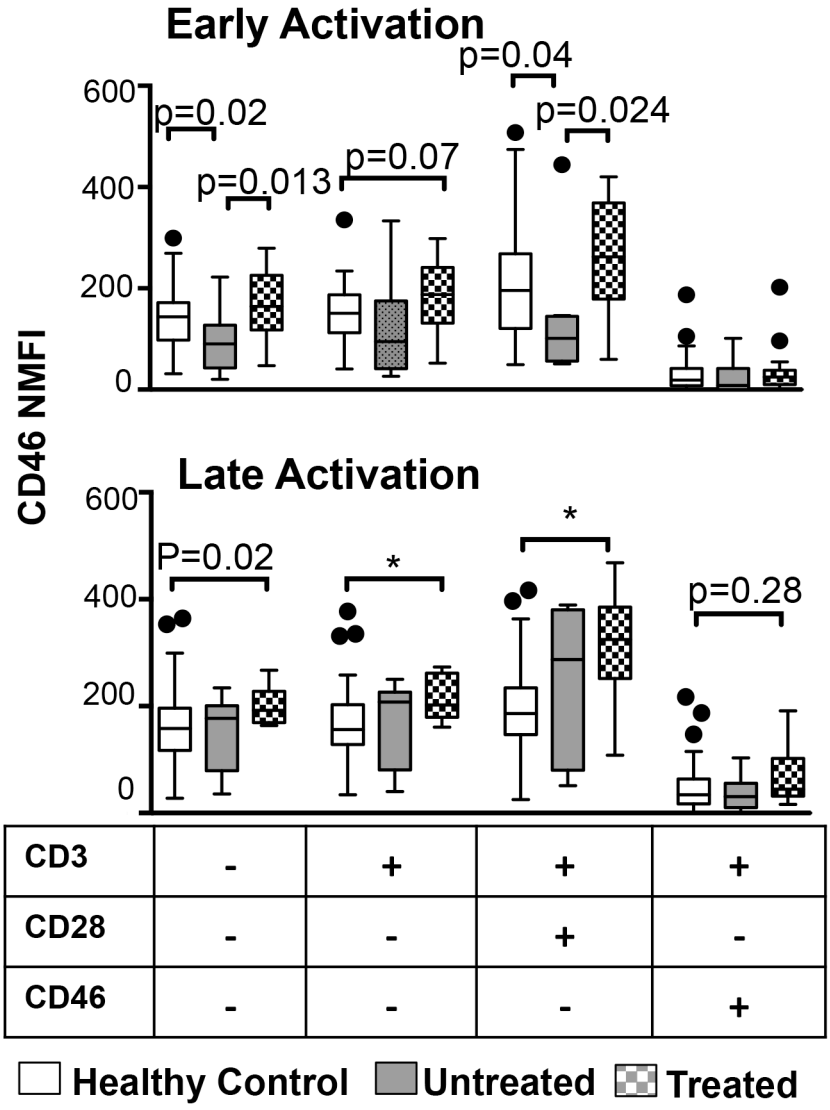


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***Figure 6.3: CD46 expression levels in treated and untreated MS patients.*** *CD46 expression was determined by flow cytometry upon activation, as indicated, in healthy controls (HC), untreated MS patients and IFNβ treated MS patients and normalised mean fluorescence intensity (NMFI) was calculated. (**Top Panel**) CD46 expression during early activation, (HC n=27, untreated MS patients n=8, treated MS patients n=13). (**Bottom Panel**) CD46 expression during late activation, (HC n=18, untreated MS patients n=7, treated MS patients n=9). A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *p≤0.01.*

### 6.4.4 Cyt1 and Cyt2 expression levels upon CD46 costimulation in MS patients

As downregulation and cleavage of Cyt1 and potentially Cyt2 is important for their function in CD46 costimulated cells (*Chapter 3*), this downregulation was assessed in $CD4^+$ T cells from MS patients. As with T cells from healthy donors, T cells from MS patients were either left unstimulated or activated with αCD3 or αCD3/CD46. The expression of Cyt1 and Cyt2 were then determined at an early activation timepoint and a late activation timepoint. As previously described, upon CD46 costimulation in healthy controls Cyt1 was downregulated at the early timepoint; however, this downregulation was not observed in MS patients (*boxed, figure 6.4*). These data could suggest that the expression of Cyt1 and its downregulation is dysfunctional in MS patients. In healthy controls, upon CD46 costimulation, Cyt2 expression was also downregulated at the late timepoint. However, upon CD46 costimulation of T cells from MS patients, there was no downregulation of Cyt2 (*boxed, figure 6.4*). Overall, therefore these data suggest that the downregulation of Cyt1 and Cyt2 may be defective in T cells from MS patients. Determining why there is no downregulation of either Cyt1 or Cyt2 upon CD46 costimulation in T cells from MS patients could shed light on the mechanism behind defective IL-10 production in MS patients. However, further experiments are required to confirm that the downregulation of Cyt1 and Cyt2 is defective in MS patients.

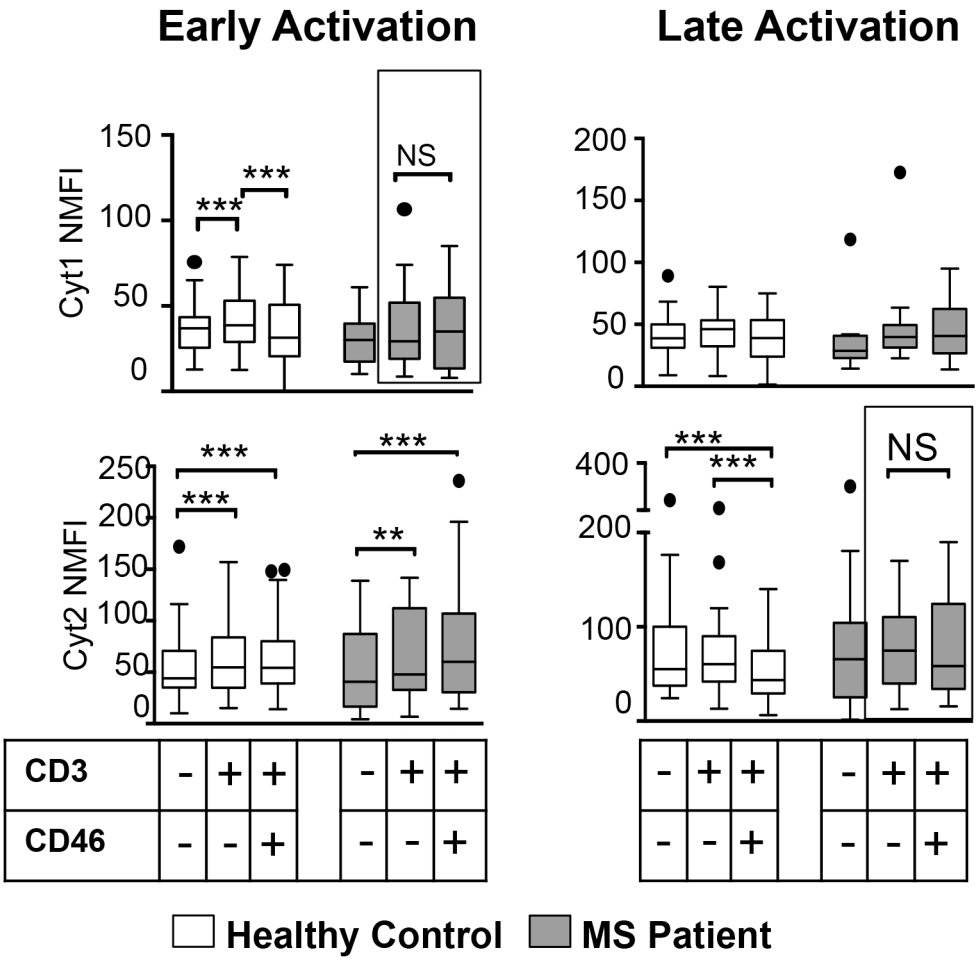


***Figure 6.4: Cyt1 and Cyt2 expression levels upon CD46 costimulation in MS patients.*** *T cells from MS patients were left unstimulated or activated in the presence of αCD3 alone or in the presence of αCD46. Cyt1 and Cyt2 expression were determined by flow cytometry during early activation (1-2 days stimulation) or late activation (4-5 days stimulation), normalised mean fluorescence intensity (NMFI) was calculated. For comparison MS CD46 expression (grey) is presented alongside expression levels previously obtained for healthy controls (HC) (white) under similar conditions (Chapter 3). (**Left Panes**) Cyt1 and Cyt2 expression during early activation (HC n=40, MS (treated and untreated) n = 17). (**Right Panel**) Cyt1 and Cyt2 expression during late activation (HC n=29, MS (treated and untreated) n = 12). A Friedman Test and Bonferroni-corrected Wilcoxon test was used for statistical analysis within the same donors, A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *** p≤0.0003 **p≤0.003, *p≤0.0167.*

### 6.4.5 Cyt1 and Cyt2 expression upon CD28 costimulation in MS patients and healthy controls

Surface expression of CD46 had an increased trend upon CD28 costimulation in MS patients compared to healthy controls, indicating that CD46 expression may be dysregulated in activated T cells from these patients. Similar to healthy controls, Cyt1 and Cyt2 expression were also analysed upon CD28 costimulation. T cells from MS patients (untreated and IFNβ treated) were left unstimulated or activated with αCD3 or αCD3/CD28 and Cyt1 and Cyt2 expression levels were determined at the early activation timepoint or the late activation timepoint. Upon CD28 costimulation, at the early and late timepoint, there was no significant difference in Cyt1 or Cyt2 expression in patients compared to healthy control (*figure 6.5*). Of note, upon CD28 costimulation there appeared to be no trend towards Cyt2 downregulation (*figure 6.5*). This could correlate with the failure in MS patients to downregulate Cyt2 upon CD46 costimulation. Thus, these data suggest that failed downregulation of Cyt2 expression in MS patients may not be limited to CD46 costimulation. Further experiments will be required to examine why Cyt1 and Cyt2 are not downregulated in MS patients as it may have important consequences for CD46 regulatory function.

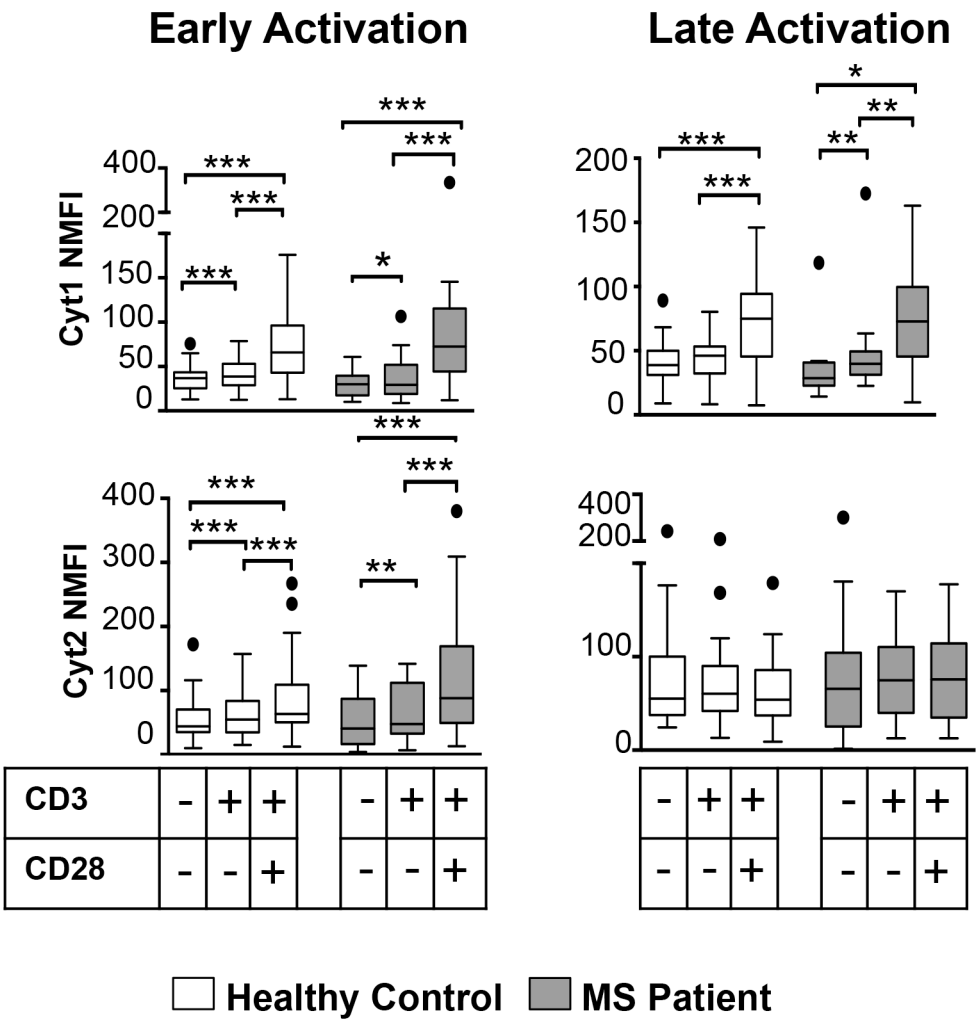


***Figure 6.5: Cyt1 and Cyt2 expression upon CD28 costimulation in CD4⁺ T cells from MS patients and healthy controls.*** *T cells from MS patients were left unstimulated or activated in the presence of αCD3 alone or in the presence of αCD28. Cyt1 and Cyt2 expression were then determined by flow cytometry at the early activation (1-2 days stimulation) or late activation (4-5 days stimulation) timepoint and the normalised mean fluorescence intensity (NMFI) was calculated. As a control, MS CD46 expression (grey) is presented alongside expression levels previously obtained for healthy controls (white) under similar conditions (Chapter 3). (**Left Panel**) Cyt1 and Cyt2 expression during early activation, (HC n=40, MS (treated and untreated) n=17). (**Right Panel**) Cyt1 and Cyt2 expression during late activation, (HC n=29, MS n=12). A Friedman Test and Bonferroni-corrected Wilcoxon test was used for statistical analysis within the same donors, A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *** p≤0.0003 **p≤0.003, *p≤0.0167.*

### 6.4.6 Normalised Cyt1 and Cyt2 expression in IFNβ-treated and untreated MS patients

The percentage increase in expression of Cyt1 and Cyt2 compared to unstimulated T cells was also calculated to assess any relative changes in Cyt1 and Cyt2 expression upon CD46 or CD28 T cell costimulation in healthy control, untreated MS patients and IFNβ treated patients. Of note, in contrast to healthy controls no downregulation of Cyt1 or Cyt2 was observed in either the untreated or treated patient groups upon CD46 costimulation (*figure 6.6*).

Upon CD46 costimulation, there was no significant differences between healthy controls and patients' Cyt1 and Cyt2 expression, however, there was an overall trend for increased Cyt1 and Cyt2 at both timepoints in patients, irrespective of treatment, compared to controls (*figure 6.6*). Upon CD28 costimulation, at the early and late timepoint, untreated patients show a trend of increased Cyt1 and Cyt2 expression compared to healthy controls (*figure 6.6*). Although not significant IFNβ treated patients also show a trend of increased levels of Cyt1 and Cyt2, upon CD28 costimulation, compared to healthy controls. However these increases are not as striking as those observed in untreated patients (*figure 6.6*).

In summary, these data indicate that upon CD46 costimulation there may be a lack of Cyt1 and Cyt2 downregulation in MS patients, irrespective of treatment. Furthermore, upon CD28 costimulation, and to a lesser extent upon CD46 costimulation, there may be increased expression of Cyt1 and Cyt2 in MS patients compared to healthy controls. This dysregulation of Cyt1 and Cyt2 expression could have important consequences for CD46 function in T cells from MS patients and importantly may contribute to defective IL-10 production. Nonetheless, it is important to stress that this is a small cohort of patient samples and further experiments need to be carried out.

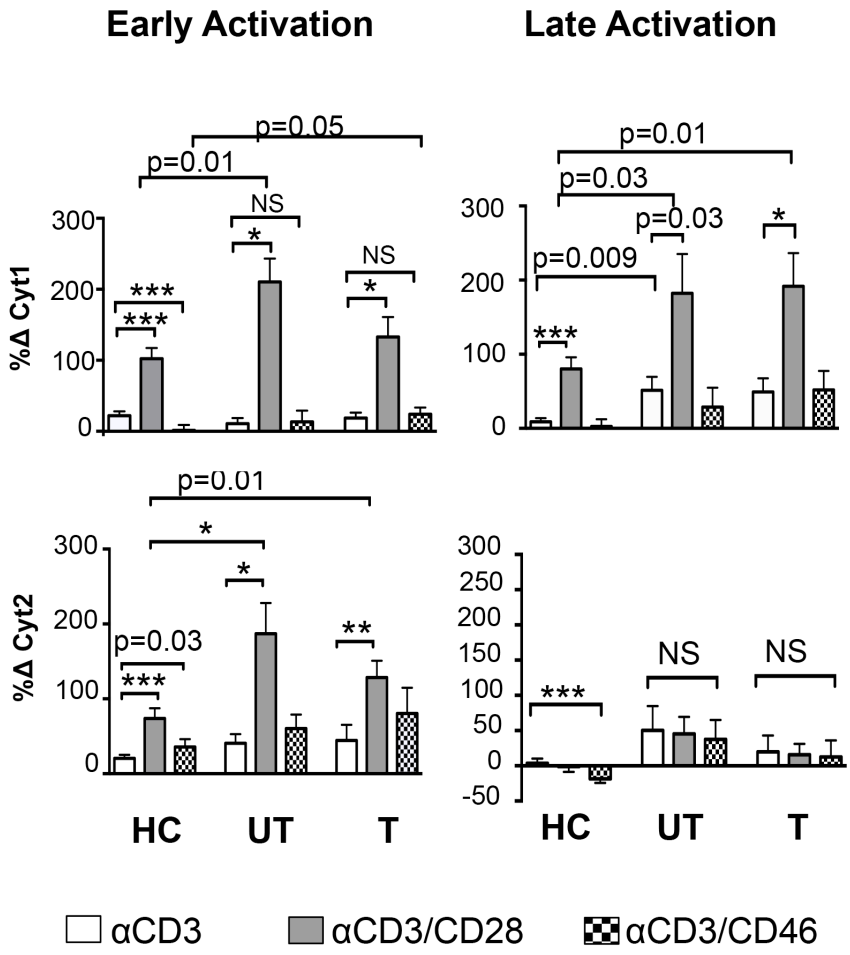


***Figure 6.6: Normalised Cyt1 and Cyt2 expression in IFNβ-treated and untreated MS patients.*** *The percentage change (%Δ) in Cyt1 or Cyt2 expression upon activation compared to unstimulated T cells was calculated based on the MFI expression determined by flow cytometry. The %Δ in expression was then compared between the three groups; healthy controls (HC), untreated MS patients (UT), and IFNβ treated patients (T). Cyt1 and Cyt2 expression at the early activation timepoint: HC n=40, UT MS patients n=9, T MS patients n=11. Cyt1 and Cyt2 expression at the late activation timepoint: HC n=29, UT MS n=6, T MS patients n=7. A Friedman Test and Bonferroni-corrected Wilcoxon test was used for statistical analysis within the same donors, *** p≤0.0003 **p≤0.003, *p≤0.0167. A Kruskal-Wallis Bonferroni-corrected Mann-Whitney test was used for statistical analysis between HC and MS cohorts, *p≤0.008.*

### 6.4.7 CXCR3 expression upon CD46 costimulation in MS patients compared to healthy controls

In order to further assess the phenotype of CD46 activated T cells in patients with MS the expression of the chemokine receptors CXCR3 and CXCR4 were assessed. Chemokine receptors can be associated with specific T cell phenotypes; CXCR3 is upregulated on Th1 type T cells, whereas CXCR4 is linked with Th2 type T cells. CXCR3 is generally upregulated in activated T cells, especially Th1 cells, whereas, CXCR4 expression is usually downregulated upon activation (Sallusto et al., 1998). Furthermore, CXCR3 has been implicated in the migration of T cells into the CNS (Balashov et al., 1999, Sorensen et al., 2002). Therefore both CXCR3 and CXCR4 expression levels were assessed in T cells from healthy controls and MS patients. MS patients consist of untreated (n=9) and treated patients (n=5) and were pooled together due to low numbers. T cells were left unstimulated or stimulated with αCD3, αCD3/CD28 or αCD3/CD46 and the percentage change in CXCR3 expression on activated T cells compared to unstimulated controls was calculated. There was no significant change in CXCR3 expression in αCD3 activated T cells or CD28 costimulated cells from MS patients compared to healthy controls. However, there was a trend for increased CXCR3 expression upon CD46 costimulation. CXCR4 expression levels were analysed similarly to CXCR3 expression; however, there was no change in CXCR4 expression under any condition examined (*figure 6.7*). Therefore, T cells from MS patients that have been costimulated with CD46 may have increased expression of CXCR3 compared to healthy controls. These data suggest that CD46 costimulated T cells from patients could preferentially migrate to the CNS and their phenotype may be skewed towards that of a Th1 T cell.

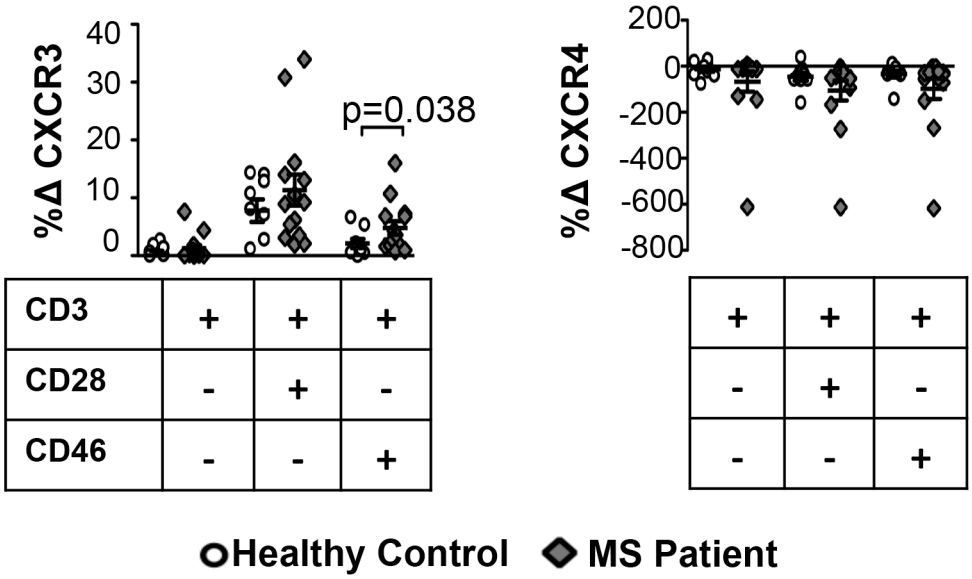


***Figure 6.7: CXCR3 expression upon CD46 costimulation in MS patients compared to healthy controls.*** *T cells from healthy controls (HC) and MS patients were activated as indicated. CXCR3 and CXCR4 expression was determined by flow cytometry. The percentage change (%Δ) in CXCR3 (left) and CXCR4 (right) upon activation compared to unstimulated T cells was calculated based on the normalised mean fluorescence intensity (NMFI), (HC n=9, MS=14). Kruskal-Wallis and Bonferroni corrected Mann-Whitney test was used for statistical analysis between HC and MS donors, *p≤0.0167.*

### 6.4.8 The $M_w$ of CD46 in activated $CD4^+$ T cells from patients with MS

Previously it was shown that upon T cell activation in healthy controls, there was a reduction in the $M_w$ of CD46 (*Chapter 5 and figure 6.8*), which reflected a decrease in CD46 glycosylation. CD46 glycosylation was also shown to play a role in the downregulation of cell surface CD46 that may also affect T cell cytokine secretion (*Chapter 5*). Therefore, differences in cell surface CD46 expression and cytokine secretion in MS patients could be the result of aberrant glycosylation in T cells from MS patients. In order to assess this, preliminary experiments were carried out to determine if there was a reduction in the $M_w$ of CD46 upon T cell activation in T cells isolated from MS patients. Under similar conditions performed to assess the $M_w$ of CD46 from healthy controls, T cells were left unstimulated or activated in the presence of αCD3, αCD3/CD46 or αCD3/CD28 for 5 days. The $M_w$ of CD46 was determined using SDS-PAGE. In contrast to healthy controls, there was no decrease in the $M_w$ of CD46 expressed on $CD4^+$ T cells from MS patients following αCD3, αCD3/CD46 or αCD3/CD28 activation (*figure 6.8*). Therefore, this suggests that the glycosylation pattern of CD46 upon T cell activation appears to be different in MS patients. This potential difference in glycosylation may alter CD46 surface downregulation and subsequent cytokine production in T cells from MS patients. However, further studies will be required before any firm conclusions can be made.

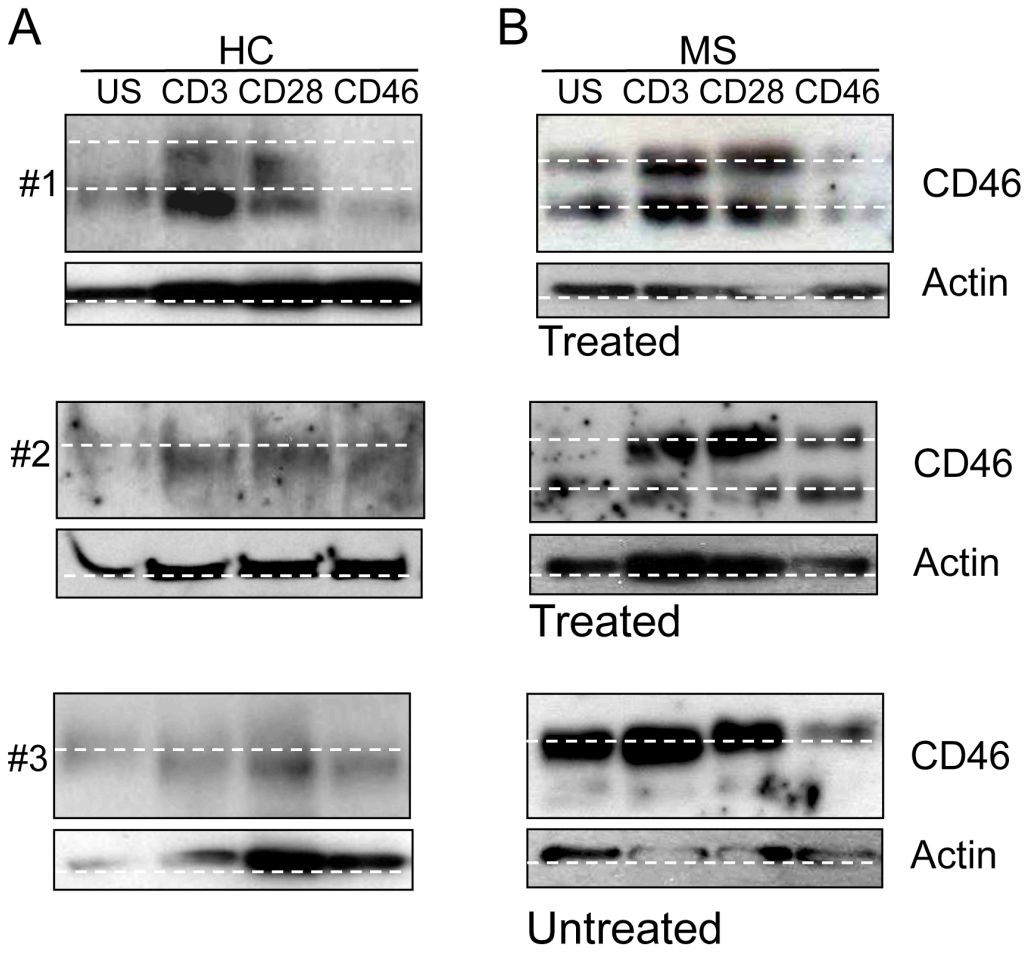


***Figure 6.8: The $M_w$ of CD46 in activated $CD4^+$ T cells from patients with MS.*** *T cells from healthy controls (HC)* ***(A)*** *or MS patients* ***(B)*** *were left US or stimulated with αCD3 alone or in the presence of αCD28 (CD28) or αCD46 (CD46) for 5 days (n=4). CD46 expression and $M_w$ were determined by SDS-PAGE. MS patients at the time of donation were untreated or receiving IFNβ treatment (treated) (Representative of 4 independent experiments).*

## 6.5 Discussion

The dysregulation of CD46 function in T cells from RRMS patients was first demonstrated by *Astier et al*. T cells costimulated with CD46 had a 4-6 fold decrease in IL-10 production compared to healthy controls, IFNγ secretion was not affected (Astier et al., 2006). The most widely used MS treatment is IFNβ, which has been shown to reduce the relapse rate and slow the course of disease in RRMS patients (Yong et al., 1998). Studies have demonstrated that IFNβ negatively regulates T cell activation, proliferation, migration across the BBB and Th1 cytokine production (Noronha et al., 1993, Stuve et al., 1996, Rep et al., 1996, Durelli et al., 2009). Moreover, IL-10 producing T cells are increased during relapse in MS patients and are also increased by IFNβ treatment (Rudick et al., 1996, Rep et al., 1999). However, in the study by *Astier et al,* IFNβ therapy did not correct the dysfunction in CD46 induced IL-10 secretion observed in RRMS patients (Astier et al., 2006). This suggests that IFNβ therapy does not act directly on CD46 induced T regulatory cells. Uncovering the mechanisms that inhibit CD46 induced IL-10 secretion could aid the development of new therapies that harness the potential of anti-inflammatory IL-10 secreting T cells. Therefore understanding the defects of CD46 induced IL-10 producing cells in MS was addressed.

***Surface regulation of CD46 expression in multiple sclerosis***

At the early activation timepoint, a trend for decreased cell surface CD46 expression was observed in unstimulated and activated T cells from untreated MS patients compared to healthy controls (*figure 6.3*). Although this observation needs to be confirmed in a larger cohort of patients it is interesting to note that increased levels of sCD46 have been observed in the serum and cerebral spinal fluid of MS patients (Soldan et al., 2001) and sCD46 has been found bound to its ligand HHV-6 in the serum of MS patients (Soldan et al., 2001, Fogdell-Hahn et al., 2005). Therefore, increased levels of sCD46 may reflect increased CD46 ligation and cleavage that correlate with decreases in CD46 surface expression. *Kemper et al* have demonstrated that CD28 activated T cells produce C3b and

provide autologous ligation of CD46 (Cardone et al., 2010). Ligation of CD46 by complement may also result in decreased CD46 expression in unstimulated cells. This also raises the question of whether a decrease in CD46 expression in untreated MS patients could reflects different subsets of T cells in MS patients compared to healthy controls. Further experiments are required to determine if this is the case. Interestingly, IL-10 production from CD46 activated $CD4^+$ T cells from asthma patients is also reduced and peripheral blood T cells from mite-sensitive asthmatics also had a decreased percentage of $CD46^+CD4^+$ T cells compared to non-asthmatic controls (Tsai et al., 2012). Therefore reduced expression of CD46 could be symptomatic of an over reactive immune system and its ligation *in vivo* by complement, pathogens or other unknown ligands. An apparently contrasting report on CD46 surface expression documented increased levels of CD46 at the RNA level in PBMCs from untreated RRMS patients (Alvarez-Lafuente et al., 2009). Nonetheless, discrepancies can be explained by differences of CD46 expression within the PBMC population or T cell population. Furthermore, T cells that undergo surface cleavage of CD46 may upregulate CD46 at the RNA levels to maintain minimal levels of CD46 surface expression in order to inhibit autologous complement activation.

Although IFNβ had no effect on CD46 induced IL-10 secretion (Astier et al., 2006), the preliminary results shown here suggest that treatment may increase CD46 surface expression (*figure 6.3*). Increased expression of CD46 may be the result of reduced MMP-9 expression, which is decreased by IFNβ treatment (Yen et al., 2010). MMP-9 is known to cleave CD46 (Cole et al., 2006) and therefore IFNβ treatment may result in decreased cleavage and increased expression of CD46. Investigating the expression levels of MMP-9 and its natural inhibitor, tissue inhibitor (TIMP)-1, upon CD46 costimulation in healthy controls and patients could shed light on why there appears to be altered expression levels of CD46 in patients. A previous report demonstrated that 44% of RRMS patients treated with IFNβ for 1 year had increased levels of CD46. Increased expression of CD46 at the mRNA level was also linked to decreased responsiveness to IFNβ

(Alvarez-Lafuente et al., 2011). As activated T cells upregulate CD46 expression (*Chapter 3*), increased expression of CD46 in unresponsive patients may reflect increased T cell activation. Determining how IFNβ upregulated CD46 expression and what role (if any) this plays in ameliorating disease remains to be determined. However, if CD46 pathways are inherently skewed towards enhancing inflammation in MS patients, inhibiting its cleavage may prevent further pro-inflammatory signalling in T cells. Interestingly, CD46 is also expressed on astrocytes and oligodendrocytes (Gasque and Morgan, 1996). If upregulation of CD46 expression upon treatment also occurs on these glial cells it could help protect them from complement attack and slow neurodegeneration.

***Cyt1 and Cyt2 expression in multiple sclerosis***

The time-dependent downregulation of Cyt1 and Cyt2 was at least partially the result of its proteolysis, which was important for the function of Cyt1 and possibly Cyt2's functions (*Chapter 3 and Chapter 4*). Upon CD46 costimulation of T cells from MS patients, irrespective of treatment, these preliminary results suggest that there is an absence of both Cyt1 downregulation during early activation and Cyt2 downregulation during late activation (*figure 6.4*). This lack of downregulation was also observed when the basal levels of Cyt1 and Cyt2 expression in unstimulated T cells were taken into account (*figure 6.6*). Further investigations are required to confirm that downregulation of Cyt1 and Cyt2 does not occur. However, it is tempting to hypothesise that the aberrant downregulation of Cyt1 and Cyt2 expression is the result of altered processing and/or localisation, which may in turn effect CD46's cytokine secretion profile. Of note, nuclear transportation of the notch ICD, the product of PγS proteolysis of Notch, is inhibited in chronic demyelinated lesions due to upregulation of the nuclear transport inhibitor protein (TIP) 3, thereby inhibiting oligodendrocyte differentiation and myelination (Nakahara et al., 2009). Therefore, a lack of Cyt1 and Cyt2 downregulation in MS patients could reflect failed transport of the Cyt1 and Cyt2 tails after proteolysis. Transportation defects could affect their signalling capacities and may also prevent their rapid degradation. Failure to

downregulate Cyt1 and Cyt2 may also be the result of increased expression at the RNA level. Indeed, an upregulation of Cyt2 mRNA was previously reported after 24 hrs CD46 costimulation in MS patients (Astier et al., 2006) and 5 days stimulation in asthma patients (Tsai et al., 2012). In MS patients, there was a general trend for increased expression of both Cyt1 and Cyt2 (relative to unstimulated T cells) upon T cell activation, compared to healthy controls, (*figure 6.6*) suggesting that T cell activation in MS patients could increase the translation of Cyt1 and Cyt2 expression. Nonetheless, time course analysis of Cyt1 and Cyt2 RNA and protein expression are required, alongside confocal analysis of Cyt1 and Cyt2 localisation, to determine the cause of deficient Cyt1 and Cyt2 downregulation in MS patients.

***CD46 and T cell migration in multiple sclerosis***

The influx of inflammatory cells across the BBB is characteristic of the pathogenesis of MS. It is well understood that chemokines and expression of their cognate receptors play a key role in directing the migration of inflammatory cells, including T cells into the CNS. Indeed, IFNβ therapy is known to modulate chemokine expression and T cell migration (Sorensen et al., 2002, Sorensen and Sellebjerg, 2002, Sorensen et al., 2004, Sellebjerg et al., 2009). CXCR3 is a chemokine receptor, characteristic of Th1 T cells that binds the chemokines CXCL9, CXCL10 and CXCL11 and is implicated in the pathogenesis of MS (Muller et al., 2010). For example, increased expression of CXCR3 in T cells is present during MS relapses (Mahad et al., 2003) and in progressive MS (Balashov et al., 1999). In addition, CXCR3 positive cells have been found in areas of plaque formation and their ligand CXCL10 is expressed by astrocytes in lesions (Balashov et al., 1999). CD46 T cell costimulation showed a trend of increased CXCR3 expression in MS patients compared to healthy controls (*figure 6.7*) and could highlight a potential role for CD46 activation in the CNS. Moreover, *Cardone et al*, have recently identified the inability of CD46 activated T cells from RA patients to switch from a Th1 phenotype to a regulatory phenotype (Cardone et al., 2010). Increased expression of CXCR3 may therefore

reflect the Th1 phenotype of CD46 activated T cell in MS and their inability to switch to an anti-inflammatory IL-10 secreting phenotype. Indeed, recent reports have highlighted the plasticity of Treg cells (Beriou et al., 2009, Zhou et al., 2009b, Voo et al., 2009, Chowdary Venigalla et al., 2012) and *Dominguez-Villar et al* have identified an increase in the frequency of Th1-like IFNγ secreting $FOXP3^+$ T cells in MS patients. These cells have a reduced suppressive capacity and are characterised by the upregulation of the Th1 transcriptor factor TBET and CXCR3 (Dominguez-Villar et al., 2011). These data suggest that the environment *milieu* may inhibit the suppressive capacity of Tregs during autoimmune diseases and promote their transition to inflammatory phenotype. Indeed, the environmental milieu may condition T cells towards a pro-inflammatory phenotype upon CD46 costimulation.

***The role of glycosylation in multiple sclerosis***

Glycosylation plays a role in CD46 surface retention and likely also affects CD46 induced cytokine production (*Chapter 5*). Therefore the glycosylation of CD46 was assessed in a small cohort of MS patients to determine if there were alterations in CD46's $M_w$ upon activation. In both treated and untreated MS patients, there was little or no decrease in CD46's $M_w$ upon CD3 stimulation suggesting that CD46 glycosylation levels may be dysregulated in MS patients (*figure 6.8*). In *Chapter 5,* decreased glycosylation upon CD3 activation promoted CD46 downregulation and cleavage. Increased surface downregulation of CD46 correlated with an increased ratio of IL-10:IFNγ. If the higher $M_w$ of CD46 reflects a higher degree of glycosylation it may attenuate CD46 downregulation and consequentially the IL-10:IFNγ ratio. In line with this hypothesis, at the late timepoint, there was a general pattern of increased CD46 surface expression in MS patients compared to healthy controls (*figure 6.2*). Furthermore there was no downregulation of Cyt1 or Cyt2 upon CD46 costimulation in MS patients, treated or untreated (*figure 6.4 and figure 6.6*). Therefore, aberrant patterns of glycosylation in T cells from MS patients could dysregulate CD46 downregulation and cytokine secretion. A key study by

*Demetriou et al* has identified Golgi N-glycosylation as the first dysregulated biochemical pathway in autoimmune diseases to account for both genetic and environmental risk factors (Mkhikian et al., 2011). This study reports how cell metabolism and vitamin D modulate N-glycosylation and can converge with MS genetic risk factors in IL-7R, IL-2R, CTLA-4 and MGAT1, a glycotransferase to regulate susceptibility to disease (Mkhikian et al., 2011). Indeed, our group has also demonstrated that vitamin D promotes CD46 down regulation and an increase in the IL-10:IFNγ ratio, upon CD46 costimulation, in healthy controls and MS patients (*manuscript in press*). IL-2 is also a key factor in regulating the CD46 Treg phenotype and the role of IL-2 in regulating CD46 glycosylation requires further investigation. As such, dysregulated N-glycosylation and environmental cues could also interfere with CD46 regulatory function in patients with MS.

It is important to emphasise that these studies examining CD46 expression and glycosylation in T cells, isolated from patients, represent a pilot study. Results obtained in small studies may give rise to skewed results based on individual donor or experimental variability. This pilot study used aged matched donors (healthy controls 35.1±7.9 and MS patients 42.5±9.5), however, there was a stronger female:male ratio in the patient cohort (healthy controls 1:1 and MS patients 3.6:1) (*Chapter 2, table 2.1*). Given the known correlation of sex with disease (Sospedra et al., 2005) the role of sex in regulating CD46 expression and glycosylation needs to be investigated in a larger group of patients. Furthermore, a larger cohort of samples from both healthy controls and patient samples, analysed in parallel, should be carried out to confirm the observations noted in this pilot study.

In summary, the plasticity of Treg cells and their dysfunction in autoimmune diseases is now well-documented (Sakaguchi et al., 2008, Costantino et al., 2008, Zhou et al., 2009a, Bluestone et al., 2009, Zhu and Paul, 2010, Lowther and

Hafler, 2012). However, the mechanisms and pathways that inhibit their suppressive capacity remain to be fully elucidated. It is likely that a combination of T cell's external cues and internal defects are responsible for maintaining or switching T cells to an inflammatory phenotype. Future studies will aim to link both functional and genetic defects in Tregs to identify dysregulated pathways in MS. As CD46 is ubiquitously expressed and given its role in regulating the innate and adaptive immune systems (Ni Choileain and Astier, 2011), CD46 is likely to play an important role in directing an integrated and appropriate inflammatory response. Understanding CD46's signalling pathways will broaden our understanding of common defective pathways that are shared amongst several autoimmune diseases.

## 6.6 Conclusions

- Preliminary results show that T cells from MS patients, irrespective of IFNβ treatment, fail to downregulate Cyt1 and Cyt2 expression upon CD46 costimulation.
- The relative increase in Cyt1 and Cyt2 expression upon CD28 and CD46 costimulation may be enhanced in both untreated and IFNβ treated MS patients compared to healthy controls.
- Preliminary results show that upon T cell activation there is no decrease in the $M_w$ of CD46 in patients with MS and this dysregulation could suggest altered levels of CD46 glycosylation in patient's T cells.

# Chapter 7 : Final Discussion and Future Directions

## 7.1 Review of the research objectives

Dysregulation of the immune system can cause chronic inflammatory diseases, such as MS, which causes physical and emotional suffering to ~2.5 million people worldwide (Sospedra and Martin, 2005, Astier and Hafler, 2007, Nylander and Hafler, 2012). Tregs are a key factor in inhibiting excessive inflammation and autoimmune diseases (Sakaguchi, 2004, Costantino et al., 2008b, Sakaguchi et al., 2010) and use of Tregs as a potential therapy is under extensive investigation (Costantino et al., 2008a, Stephens et al., 2009, Sabatos-Peyton et al., 2010, Bluestone, 2011, Lowther and Hafler, 2012). The primary focus of this thesis was to address the expression, regulation and function of the T cell costimulatory molecule, CD46. Interest in the regulation of CD46's expression and function was driven by its role in promoting a T regulatory phenotype, characterised by the secretion of IL-10 (Kemper et al., 2003), and because that pathway was defective in MS (Astier et al., 2006). Evidence from a transgenic mouse model expressing one of the CD46 isoforms, Cyt1 or Cyt2 demonstrated that CD46's isoforms had contrasting roles in regulating inflammation (Marie et al., 2002). This led to the hypothesis that Cyt1 and Cyt2 could have distinct functions in human T cells and that dysregulation of their expression and/or processing could contribute to the lack of IL-10 secretion observed in MS patients. Thus, the specific aims of this work were to elucidate: (1) the expression levels of cell surface CD46 and intracellular Cyt1 and Cyt2 during T cell activation in healthy controls; (2) the function of the cytoplasmic tails, Cyt1 and Cyt2 during human T cell activation in healthy controls; (3) the mechanisms of the regulation of CD46 expression; and (4) to determine if defective expression or processing of CD46 was responsible for limited IL-10 production in CD46 costimulated T cells from MS patients. These points were addressed and their

contributions to our understanding of CD46's function in T cell activation are discussed below.

## 7.2 The main contribution of this thesis

The expression levels of surface CD46 and/or its isoforms, Cyt1 and Cyt2, had not been previously assessed throughout the activation of human primary T cells. It has been established that CD46 expression levels are tightly regulated during T cell activation (*Chapter 3*). Proteolytic cleavage of CD46 by MMP/ADAM(s) had been documented in apoptotic neuronal and epithelial cells (Elward et al., 2005, Cole et al., 2006, Hakulinen and Keski-Oja, 2006), during the incubation of various cell types with Loxoceles' venom (Van Den Berg et al., 2002) and upon the ligation of CD46 in epithelial cells with *Neisseria* (Weyand et al.). Proteolysis of CD46 was known to play a role in regulating complement induced cell death (Elward et al., 2005, Cole et al., 2006, Hakulinen and Keski-Oja, 2006), however CD46 proteolysis in human T cells had not been addressed. Importantly, this study was the first to report a function of CD46 proteolysis in T cell activation (Ni Choileain et al., 2011). Upon CD46 costimulation of T cells, surface CD46 expression was downregulated and this was at least partially the result of cleavage of the extracellular domain by MMP/ADAM(s) (*Chapter 4*). There was also specific regulation of the expression of CD46 isoforms, Cyt1 and Cyt2, during T cell activation. Upon CD46 costimulation, Cyt1 expression was downregulated after 1-2 days activation, whereas Cyt2 expression was downregulated after 4-5 days stimulation. The time-dependent downregulation of Cyt1 and Cyt2 was at least partially the result of PγS cleavage (*Chapter 4*). MMP/ADAM and PγS cleavage of type I transmembrane proteins has been extensively studied for proteins such as Notch (Bray, 2006, Parks and Curtis, 2007, Fortini, 2009). Proteolysis of Notch's extracellular domain by MMPs, followed by its intracellular cleavage by PγS, facilitate the release of an ICD, which trafficks to the nucleus via the endosomal pathway. Nuclear translocation of the Notch ICD allows direct transcriptional regulation of target genes (Bray, 2006, Parks and Curtis, 2007, Fortini, 2009). Indeed, CD46 is also downregulated

through an endosomal pathway upon CD46 costimulation (*chapter 3*) and proteolytic cleavage of Cyt1 was important for its function (*Chapter 4*). Cyt1 promotes T cell activation and this function is dependent on its cleavage by PγS. Cyt2 was also shown to promote T cell proliferation when expressed in a full-length CD19-Cyt2 form but not when expressed in a smaller CTF protein. These results suggest that Cyt2's function in regulation T cell activation may be dependant on it's proteolysis and/or its cellular location at the membrane, however, further experiments are required to confirm this conclusion (*Chapter 4*). These results also established that Cyt1 promotes an increased IL-$10^+$:IL-$10^+$ IFN$\gamma^+$ secretion ratio. In accordance with Kemper et al (Cardone et al., 2012), these data demonstrate that Cyt1 is therefore important in promoting a more regulatory like T cell phenotype. Importantly, proteolytic cleavage of Cyt1 and potentially Cyt2 is an important factor in regulating their function.

Determining how CD46 proteolysis was regulated led to the investigation of the TCR's role in regulating CD46 T cell activation. Indeed, TCR activation alone increased CD46 expression (*Chapter 3*) and decreased the level of CD46 glycosylation (*Chapter 5*). Upon CD46 costimulation, decreased glycosylation correlated with increased levels of sCD46 suggesting that lower levels of CD46 glycosylation could promote CD46 cleavage by MMP/ADAM(s) (*Chapter 5*). These novel data implicating the TCR in regulating the expression and cleavage of CD46 highlights the inherent role of CD46 during T cell activation.

The role of glycosylation in regulating CD46's expression and function was further addressed using glycosylation inhibitors and mutant CD46 proteins that lacked specific glycosylation sites. These investigations suggested that the glycosylation of CD46 not only regulates its surface expression but also the IL-10 and IFNγ secretion profile. O-deglycosylation attenuated CD46 downregulation and the ratio of IL-10:IFNγ secretion. Conversely, deglycosylation of the N-glycans at the SCR1 and/or SCR4 domain may promote CD46 surface

downregulation and an increased the IL-10:IFNγ ratio (*Chapter 5*). The role of CD46 glycosylation was known to regulate CD46's function as a complement (Liszewski and Atkinson, 1996, Liszewski et al., 1998) and pathogen receptor (Maisner et al., 1996, Kallstrom et al., 2001) but had not be addressed in CD46's capacity as a costimulatory receptor. Therefore, alterations in CD46 glycosylation levels offer a novel mechanism whereby CD46 may regulate inflammatory and anti-inflammatory T cell responses. Glycosylation levels of proteins are dependent on the metabolic state, environmental *milieu* and cell activation (Wagers et al., 1998, Grabie et al., 2002, Comelli et al., 2006, Toscano et al., 2007, Mkhikian et al., 2011, Grigorian et al., 2012). Thus, alterations in CD46 glycosylation could also be responsive to soluble factors in the environmental *milieu* such as IL-2 or Vitamin D, both of which have previously been shown to regulate CD46's T cell function (Kemper et al., 2003, Cardone et al., 2010) (*manuscript in press*).

In light of the data gathered in this thesis and literature available on other type 1 membrane receptors, a model for CD46 processing is proposed: TCR activation (1) induces deglycosylation or expression of lower glycosylated forms of CD46 (2); Lower glycosylated CD46 is more susceptible to proteolytic cleavage, which occurs either at the membrane or after endocytosis (3); After cleavage, CD46's ICDs can have unique signalling capacities that could involve gene transcription in the nucleus (4); CD46's ICDs are degraded to attenuate signalling prior to/post nuclear translocation (5) (*figure 7.1*).

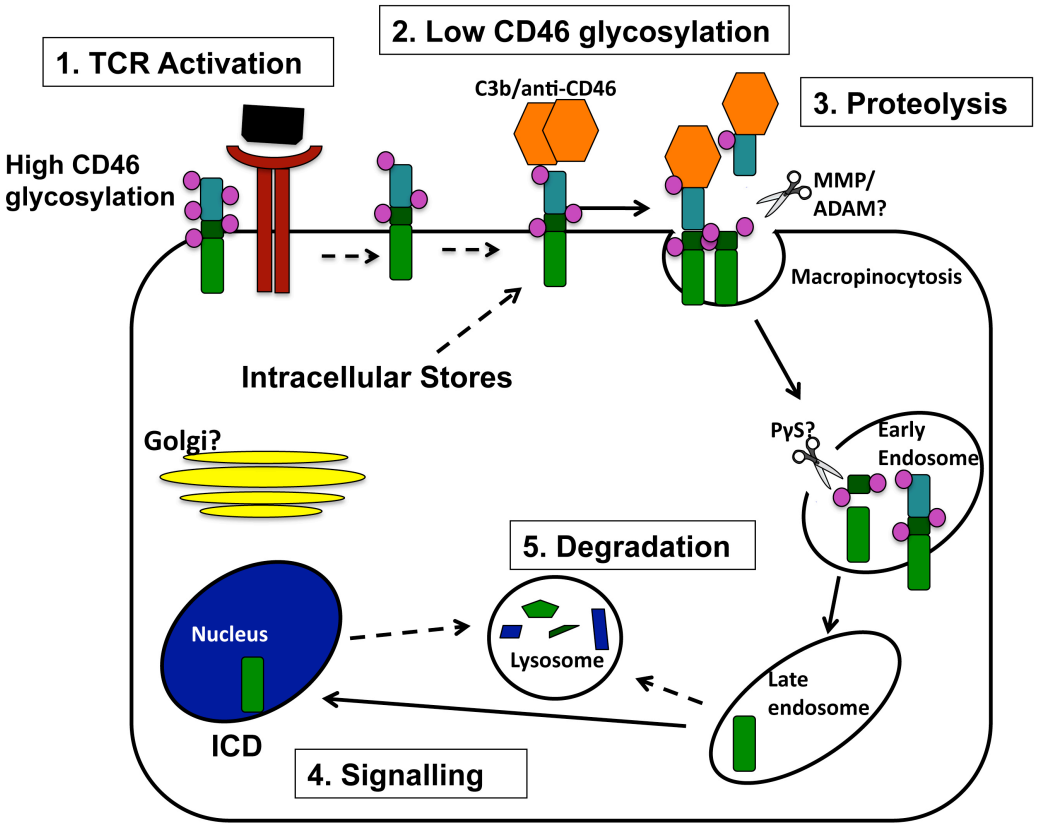


***Figure 7.1: Proposed model of CD46 processing and signalling.***

Understanding the regulation of CD46 expression and processing in healthy controls is fundamental to understanding CD46 dysfunction during inflammatory disease. In an effort to understand why there was defective IL-10 production upon CD46 costimulation in T cells from MS patients, the expression levels of CD46, Cyt1 and Cyt2 were analysed in comparison to healthy controls. In healthy controls, the time-dependent downregulation of Cyt1 and Cyt2 by PγS was observed. In T cells from RRMS patients, there was no downregulation of Cyt1 or Cyt2, irrespective of IFNβ treatment. Indeed, in the original study by *Astier et al* IFNβ treatment did not restore IL-10 secretion in CD46 costimulated T cells (Astier et al., 2006). Although the role of Cyt1 and Cyt2 downregulation in regulating cytokine secretion remains to be determined, a lack of Cyt1 downregulation, which promotes IL-10 secretion, could also be partially responsible for limited IL-10 secretion in MS patients. The functional outcome of

failed Cyt2 downregulation warrants further investigation. However, considering the role of Cyt2 in regulating T cell proliferation, it could promote prolonged T cell activation and inflammatory responses in MS patients. Failure to downregulate Cyt1 and Cyt2 could be the result of the following defects (1) PγS cleavage (2) transport and degradation of ICDs (3) dysregulated glycosylation of CD46 (4) increased Cyt1 or Cyt2 protein translation in MS patients compared to healthy controls. Further experiments are required to determine if one or more of these hypothesis are correct and if they contribute to reduced IL-10 secretion from CD46 activated T cells in MS (*discussed below*). Notably, preliminary results suggest that TCR activation in MS patient does not decrease the $M_w$ of CD46 (*Chapter 5*) and support the hypothesis that dysregulated glycosylation of CD46 in MS patients could contribute to a lack of isoform downregulation and dysregulated cytokine secretion.

## 7.3 Future work

Further investigations into CD46 processing will pave the way for increased understanding of the defects observed in MS patients and other autoimmune diseases. Future work will focus on (1) the localisation of CD46 and CD46's ICDs upon activation; (2) glycosylation of CD46 during T cell activation; (3) the kinetics of CD46 expression in specific T cell subsets.

***Localisation of CD46 and CD46's ICD***

To further elucidate CD46's signalling pathways it is key to understand what happens to the ICD fragments after their release. Preliminary studies suggested that both Cyt1 and Cyt2 translocated to the nucleus upon CD46 costimulation (*Chapter 4*), which implies that Cyt1 and Cyt2's ICDs could regulate gene transcription, for example, ChIP-sequencing assays may confirm direct links with Cyt1 and Il-10 related genes. Defects in the transport of the Notch ICD to the nucleus have already been documented in MS patients (Nakahara et al., 2009) and suggest that CD46 ICD could be trapped in the cytoplasm. This would explain why no downregulation of Cyt1 or Cyt2 was observed in MS patients upon CD46 costimulation. If the Cyt1-ICD or the Cyt2-ICD could not translocate to the nucleus it could partially explain why there was reduced IL-10 secretion upon CD46 costimulation in T cells from patients with MS. Detailed confocal microscopy studies are required to examine CD46's ICD trafficking after cleavage and would include fluorescent labelling of early, late and recycling endosomes in addition to nuclear and lysosomal markers. These studies may confirm localisation of Cyt1 and Cyt2 ICDs within the nucleus. In order to determine a potential defect in translocation in MS patients, the expression levels of proteins involved in the transport of ICDs e.g. the transport inhibitor TIP30 whose expression is dysregulated in MS (Nakahara et al., 2009), would also be examined. Expression levels of these proteins would be examined at the RNA and protein level using PCR, SDS-PAGE and/or flow cytometry upon T cell

activation in both healthy controls and MS patients. These studies would determine if CD46 ICD trafficking is defective in MS patients.

***Glycosylation of CD46 during T cell activation***

Detailed mass spectronomy analysis of CD46 glycosylation in unstimulated, TCR activated and CD46 costimulated T cells in a cohort of both healthy controls and RRMS patients will shed light of the exact changes of CD46 glycosylation during T cell activation. Alterations in glycosylation levels would also be determined in the presence of vitamin D and IL-2, as these factors are known to regulate CD46 cytokine secretion. *Mkhikian et al*, have recently identified how the convergence of genetics (e.g. an IL-2 receptor variant) and vitamin D levels can result in the dysregulation of the N-glycosylation pathway in MS patients (Mkhikian et al., 2011). Studies investigating CD46 glycosylation would further develop our understanding of the role of glycosylation in autoimmune diseases.

***Expression level kinetics of CD46 in specific T cell subsets***

$CD4^+$ T cells are a heterogeneous group of cells that include naïve and memory effector T cells alongside various subtypes of Tregs. To address the question of whether dysregulated expression of Cyt1 and Cyt2 is due to differences in different T cell subsets between healthy controls and MS patients, kinetic studies of Cyt1 and Cyt2 expression over the 5-day period of activation need to be carried out in the different T cell populations. Expression levels would be assessed at the protein level using flow cytometry and at the RNA level using PCR. Assessing the RNA levels alongside the protein levels would indicate if increased expression of Cyt1 and Cyt2 compared to CD3 activated T cells was the result of increased protein translation or post-translation processing.

## 7.4 Concluding Remarks

By examining CD46 expression levels and processing in healthy controls and MS patients, it is possible to determine why there is defective IL-10 production in patients with MS and other autoimmune diseases. The field of glycosylation is complex and its role in autoimmune disease is only beginning to emerge. Understanding how CD46's glycosylation phenotype adapts to the environmental *milieu* may offer an explanation as to how T cells regulate their function in a changing immunological environment. Moreover, CD46 expression is ubiquitous and its functions are diverse during immunological responses. Therefore unravelling the mechanisms of CD46 processing could have wide ranging implications for the study of the human immune system. This thesis has advanced our knowledge of CD46 function in T cells and has raised questions about how CD46 processing can regulate T cell plasticity in health and disease.

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# Appendix: Published Papers

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PLoS one

# The Dynamic Processing of CD46 Intracellular Domains Provides a Molecular Rheostat for T Cell Activation

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## Abstract

***Background:*** Adequate termination of an immune response is as important as the induction of an appropriate response. CD46, a regulator of complement activity, promotes T cell activation and differentiation towards a regulatory Tr1 phenotype. This Tr1 differentiation pathway is defective in patients with MS, asthma and rheumatoid arthritis, underlying its importance in controlling T cell function and the need to understand its regulatory mechanisms. CD46 has two cytoplasmic tails, Cyt1 and Cyt2, derived from alternative splicing, which are co-expressed in all nucleated human cells. The regulation of their expression and precise functions in regulating human T cell activation has not been fully elucidated.

***Methodology/Principal Findings:*** Here, we first report the novel role of CD46 in terminating T cell activation. Second, we demonstrate that its functions as an activator and inhibitor of T cell responses are mediated through the temporal processing of its cytoplasmic tails. Cyt1 processing is required to turn T cell activation on, while processing of Cyt2 switches T cell activation off, as demonstrated by proliferation, CD25 expression and cytokine secretion. Both tails require processing by Presenilin/γSecretase (P/γS) to exert these functions. This was confirmed by expressing wild-type Cyt1 and Cyt2 tails and uncleavable mutant tails in primary T cells. The role of CD46 tails was also demonstrated with T cells expressing CD19 ectodomain-CD46 C-Terminal Fragment (CTF) fusions, which allowed specific triggering of each tail individually.

***Conclusions/Significance:*** We conclude that CD46 acts as a molecular rheostat to control human T cell activation through the regulation of processing of its cytoplasmic tails.

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## Introduction

Proper functioning of the immune system depends not only on a rapid, effective activation of immune cells, but also on timely downregulation of the response. Inadequate termination of these responses could lead to autoimmunity, chronic inflammation and cancer. Though the parameters of T cell activation are well documented, mechanisms that participate in T cell contraction are less well characterized. A number of mechanisms have been reported (and recently compiled in a series of reviews [1]). These include regulation of cell death [2] and autophagy [3], upregulation of negative signaling molecules such as CTLA-4 [4] and PD-1 [5], metabolic amino-acid regulation [6,7], control by T regulatory (Treg) cells [8] and Treg induction by dendritic cells [9], among many others. Thus, homeostasis of the immune system depends on a fine balance between immune cell induction and deactivation.

CD46 was first identified as a regulator of the complement cascade [10,11], but has subsequently been shown to link innate immunity to acquired immunity. Its activation promotes T cell activation and differentiation. Costimulation of TCR with CD46 leads to increased T cell proliferation [12], and affects T cell morphology [13] and polarity [14]. Furthermore, CD46 activation leads to Tr1 Treg differentiation [15]. This was characterized by secretion of high amounts of IL-10 [15] and granzyme B [16]. Interestingly, a recent report demonstrates that CD46 can in fact switch T cell differentiation from a Th1 to a Tr1 phenotype, depending on IL-2 concentrations present in the milieu [17]. This underlines the importance of the plasticity of CD46 in controlling T cell activation. We have previously shown that Tr1 differentiation is altered in patients with multiple sclerosis (MS). IL-10 secretion upon CD3/CD46 costimulation was impaired in T cells from ~50% of patients with MS [18,19]. The lack of Tr1 differentiation in MS was recently confirmed by another study

[20] and in a primate model of MS [21], and the dysregulation of CD46 pathways in T cells was recently described in patients with asthma [22] and with rheumatoid arthritis [17]. The role of CD46 in human diseases highlights its importance in controlling T cell activation, and further underlines the need to understand its regulation and the molecular mechanisms responsible for its functions.

CD46 is a type I membrane protein expressed in all nucleated human cells. Its isoforms, products of alternative splicing, have four complement control repeats (CCR) at the N-terminus, followed by a heavily glycosylated region rich in serine, threonine and proline, a transmembrane segment, and one of two short cytoplasmic tails termed Cyt1 and Cyt2 [23]. Both tails can transmit signals [24,25]. Most cell types co-express Cyt1 and Cyt2 except for brain and kidney cells, which predominantly express Cyt2 [26], and their function is mostly unknown. As mice do not express CD46 except for testis, we initially studied their role in inflammation in a CD46 transgenic mouse model of T cell-dependent contact hypersensitivity. We reported that CD46-Cyt1 inhibits inflammatory responses, whereas Cyt2 augments inflammation [27]. We also demonstrated that CD3/CD46 coactivated T cells from MS patients have higher levels of CD46-Cyt2 mRNA compared to activated T cells from healthy donors [19]. This suggests that the higher level of CD46-Cyt2 transcript resulting from CD46 engagement in MS patients may influence their T cell responses. Recently CD46 was shown to be a substrate for the presenilin/γ-secretase (P/γS). Upon infection by pathogenic *Neisseria*, epithelial cell CD46 was sequentially cleaved by MMP and P/γS [28]. MMP cleavage releases a soluble ectodomain and a C-Terminal Fragment (CTF) consisting of the transmembrane region and cytoplasmic tail. P/γS then cleaves the CTF, releasing the Cyt1 and Cyt2 tails into the cytosol. Whether the Cyt1 and/or Cyt2 ICDs have biological activity is currently unknown.

Herein, we investigated the regulation of CD46 expression upon T cell activation and tested the hypothesis that P/γS modulates the function of CD46-Cyt1 and CD46-Cyt2 on immune function. We demonstrate a novel function of CD46 in terminating T cell activation. We first present evidence of CD46 processing in human primary T cells. We show that CD46 is cleaved by MMP in CD46-coactivated human T cells. Furthermore, our data illustrate that Cyt1 and Cyt2 levels fluctuate dynamically during T cell stimulation. CD28 costimulation results in an increase in Cyt1 and Cyt2 expression, suggestive of a crosstalk between CD46 and CD28. However, upon CD46 coactivation, CD46 cytoplasmic isoforms were temporally downregulated. Cyt1 expression decreased transiently, whereas Cyt2 expression increased then strongly decreased. Addition of P/γS enzymatic complex inhibitors impaired CD46 tail downregulation. We demonstrate the requirement of CD46 CTF processing in immune regulation by two approaches. First, we expressed uncleavable mutant CD46 Cyt1 and Cyt2 CTF constructs (hereafter called CTF1 and CTF2, respectively) in primary human T cells. Expression of wild-type (wt) CTF1 promoted T cell proliferation, CD25 expression and IL-10 secretion, whereas expression of uncleavable CTF1 (UNCL.F1) abrogated T cell activation, demonstrating that cleavage of Cyt1 is required for its function. Expression of wt CTF2 decreased IFNγ secretion, while expression of uncleavable CTF2 (UNCL.F2) enhanced T cell proliferation, increased CD25 expression and IFNγ secretion, indicating that Cyt2 cleavage acts as an inhibitory signal for T cell activation. Second, we expressed CD19 ectodomain-CD46 CTF fusion proteins in primary T cells. Triggering of Cyt1 or Cyt2 by CD19 ligation led to similar conclusions in terms of cytokine production and proliferation. Taken together, our data indicate that processing of CD46 tails is required to first promote T cell activation followed by signals resulting in T cell inhibition, demonstrating the unexpected role of CD46 in turning off its own activation in a negative feedback loop. These data suggest that the timely activity of P/γS on the two CD46 isoforms provides a molecular rheostat for regulating T cell activation.

## Results

### CD46 is cleaved by a metalloproteinase upon T cell activation

We first assessed whether activating primary human T cells via CD46 could modulate its surface expression. Purified human $CD4^+$ primary T cells were activated by immobilized anti-CD3, anti-CD3 and anti-CD28 (anti-CD3/CD28), or anti-CD3 and anti-CD46 antibodies (CD3/CD46) for 2 days. The presence of CD46 ectodomain on T cells was monitored by flow cytometry. CD3 and CD3/CD28 costimulation led to an increase in surface CD46 levels (Figure 1A). In contrast, CD3/CD46 stimulation resulted in a loss of surface CD46. Reduced levels of surface CD46 were observed up to 5 days post-activation (Figure 1B).

In order to determine whether MMPs are involved in the downregulation of surface CD46, we cultured T cells in presence of GM6001, a broad MMP inhibitor. Activating T cells with CD3/CD46 in the presence of GM6001 partly restored surface CD46 levels (Figure 1C). Moreover, using an ectodomain-specific antibody, we were able to immunoprecipitate CD46 from supernatants of CD3/CD46 stimulated cells, but not from supernatants of unstimulated cells, or cells stimulated with CD3 or CD3/CD28 (Figure 1D). The slightly lower molecular weight of soluble CD46 compared to membrane CD46 is the size predicted for the ectodomain released by MMP. Furthermore, addition of GM6001 decreased the levels of soluble CD46 in the supernatants of CD3/CD46 activated T cells. Hence, T cell activation via CD46 causes its ectodomain to be released from the membrane, and MMP cleavage is responsible at least in part for this shedding. We next determined whether addition of MMP inhibitor could modulate CD46 function and notably IL-10 production. Addition of GM6001 slightly increased the proliferation of CD46-activated T cells. However, it significantly inhibited IL-10 production (Figure S1). These data suggest that CD46 processing may be required for IL-10 production by CD46-activated T cells.

### P/γS causes fluctuations in the levels of the two CD46 cytoplasmic tails

We next addressed the possibilities of further downstream processing of CD46 cytoplasmic tails in primary human $CD4^+$ T cells. $CD4^+$ primary T cells were activated by anti-CD3/CD46 antibodies for ~28–40 hrs (early time point) or 96–120 hrs (late time point). CD46 tails Cyt1 and Cyt2 were monitored by flow cytometry using tail-specific monoclonal antibodies [29]. At the early time point, Cyt1 levels were reduced in stimulated cells compared to unstimulated cells ($p = 0.026$), whereas Cyt2 levels were significantly increased ($p = 0.0002$; Figure 2A and Figure S2). At the late time point, Cyt1 levels in stimulated and unstimulated cells were equivalent, whereas Cyt2 levels were significantly lower than those in unstimulated cells (Figure 2A and Figure S2). Stimulating cells with CD3 or CD3/CD28 antibodies, i.e., without CD46 ligation, slightly increased cytoplasmic Cyt1 at the early time point, with CD3/CD28 having the most dramatic effect ($p = 0.0001$; Figure 2B). Cyt1 levels remained elevated at the late time point, but only in CD3/CD28 stimulated cells ($p = 0.0006$). In contrast, CD3 and CD3/CD28 stimulated cells had increased Cyt2 levels only at the early time point, with CD3/CD28

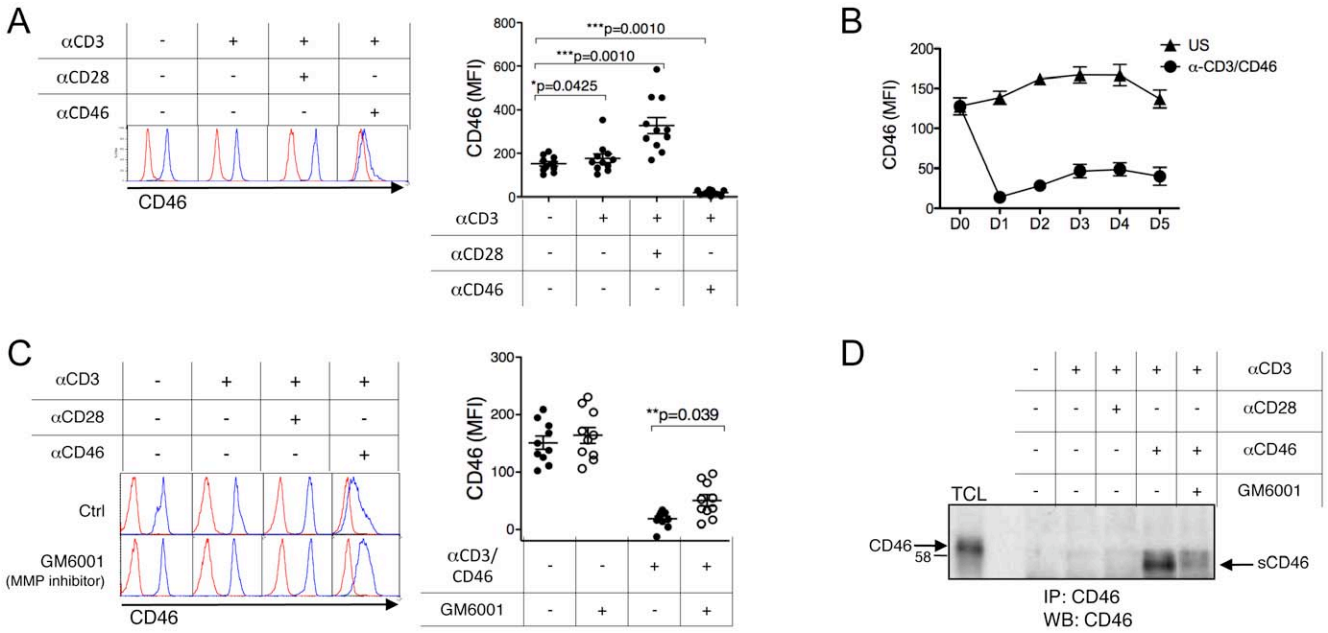


**Figure 1. CD46 ectodomain is processed by MMPs upon T cell activation.** (**A**) $CD4^+$ T cells were left unstimulated, or stimulated as indicated with immobilized anti-CD3, anti-CD3/CD28 or anti-CD3/CD46 for 48 hrs. The expression of CD46 at the cell surface was examined by flow cytometry. The results obtained for one donor are shown. The normalized MFI for CD46 (Δ to the isotype control) are plotted for the different donors analyzed (mean ± SEM, n = 12). All data were analyzed using the Wilcoxon test, a non-parametric paired t-test that does not assume Gaussian distribution. (**B**) $CD4^+$ T cells were activated with immobilized anti-CD3/CD46 antibodies or left unstimulated (US) for several days and CD46 surface expression monitored daily. (**C**) $CD4^+$ T cells were activated with immobilized antibodies as indicated in presence of GM6001, a broad metalloproteinase inhibitor (10 μM), or DMSO as a control. After 2 days, the cell surface expression of CD46 was determined by flow cytometry. The representative plots obtained for one donor are shown, and the normalized data obtained for the different donors (n = 10) are shown on the right panels. (**D**) The presence of sCD46 in the cell culture supernatants of activated T cells, as indicated, was determined after CD46 immunoprecipitation and western-blot analysis. TCL = Total Cell Lysate, as a control for membrane CD46. Representative of 2 experiments.
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stimulation having again the most dramatic effect ($p = 0.0001$). These data demonstrate that T cell costimulation by CD46 but also by CD28 regulates the levels of expression of CD46 cytoplasmic tails, and suggest a crosstalk between these two costimulatory molecules.

We next investigated whether P/γS is involved in regulating Cyt1 and Cyt2 tail levels. $CD4^+$ T cells were activated by immobilized anti-CD3/CD46 antibodies in the presence of DAPT, a P/γS pharmacological inhibitor, or of DMSO as a control, for 2 or 4 days, and Cyt1 and Cyt2 levels were analyzed by flow cytometry using tail-specific monoclonal antibodies. A representative experiment with one set of donor cells is presented in Figure 2C. Both Cyt1 and Cyt2 levels were increased in the presence of DAPT, compared to cells stimulated in the absence of DAPT (Figure 2C). An increased expression of Cyt1 was detected at day 2 while increased expression of Cyt2 was detected at day 4. Similar results were obtained when the experiment was repeated with addition of L-685,458, another P/γS inhibitor (Figure S3). These inhibitors had no effect on the levels of surface CD46 (not shown). These data suggest that P/γS activity modulates the levels of Cyt1 and Cyt2 isoforms in T cells, in a time-dependent fashion.

## IL-2 has no significant effects on CD46 expression

CD46 costimulation drives Tr1 differentiation in presence of IL-2 [15,17]. Hence, we assessed whether IL-2 modulates CD46 expression. $CD4^+$ T cells were activated with anti-CD3/CD46 antibodies in presence of increasing doses of IL-2. Expression of cell surface CD46 and of its cytoplasmic isoforms were monitored overtime by flow cytometry (Figure 3). No significant effect was observed for surface CD46 expression (Figure 3A). Although there was a small increase in Cyt1 and Cyt2 expression in presence of IL-2, it was not significant (Figure 3B). Hence, IL-2 did not seem to considerably affect CD46 expression.

## The expression of the two CD46 CTFs differently affects T cell responses

To further investigate the role of P/γS processing of CD46 in T cell functions, we next expressed the Cyt1 or Cyt2 CTF in primary T cells. These constructs consist of the membrane-spanning segment of CD46 and either the Cyt1 or Cyt2 cytosolic tail. Hereafter, these constructs are named CTF1 (containing the Cyt1 tail) or CTF2 (containing the Cyt2 tail) (Figure 4). We first checked that primary T cells transfected with empty vector responded normally to CD46 and CD28 co-stimulation. Indeed, these cells produced high levels of IL-10 and low levels of IFNγ when activated by CD46 antibodies, and higher levels of IFNγ when activated by CD28 antibodies. As reported, cells activated with CD28/CD46 produced more IL-10 than those activated by CD28 alone (Figure 5A and [15]). Cells were then transfected with plasmids encoding CTF1, CTF2 or with empty vector (CVO). Twenty-four hours post-transfection, the cells were activated with anti-CD3/CD28 or anti-CD3/CD46, or with anti-CD3/CD28/CD46, and after 4 days, IL-10 and IFNγ secretion was assessed by flow cytometry. We determined the proportion of $IL\text{-}10^+$, $IL\text{-}10^+IFN\gamma^+$ and $IFN\gamma^+$ secreting cells after expression of each construct and calculated the proportion of $IL\text{-}10^+/IL\text{-}10^+IFN\gamma^+$ and of $IFN\gamma^+/IL\text{-}10^+IFN\gamma^+$ to detect the potential effects of the CTFs on specific cytokine production (Figure 5B). Activation of CTF1-expressing cells by CD3/CD28 and CD3/CD28/CD46 increased the proportion of IL-10-secreting cells in the population

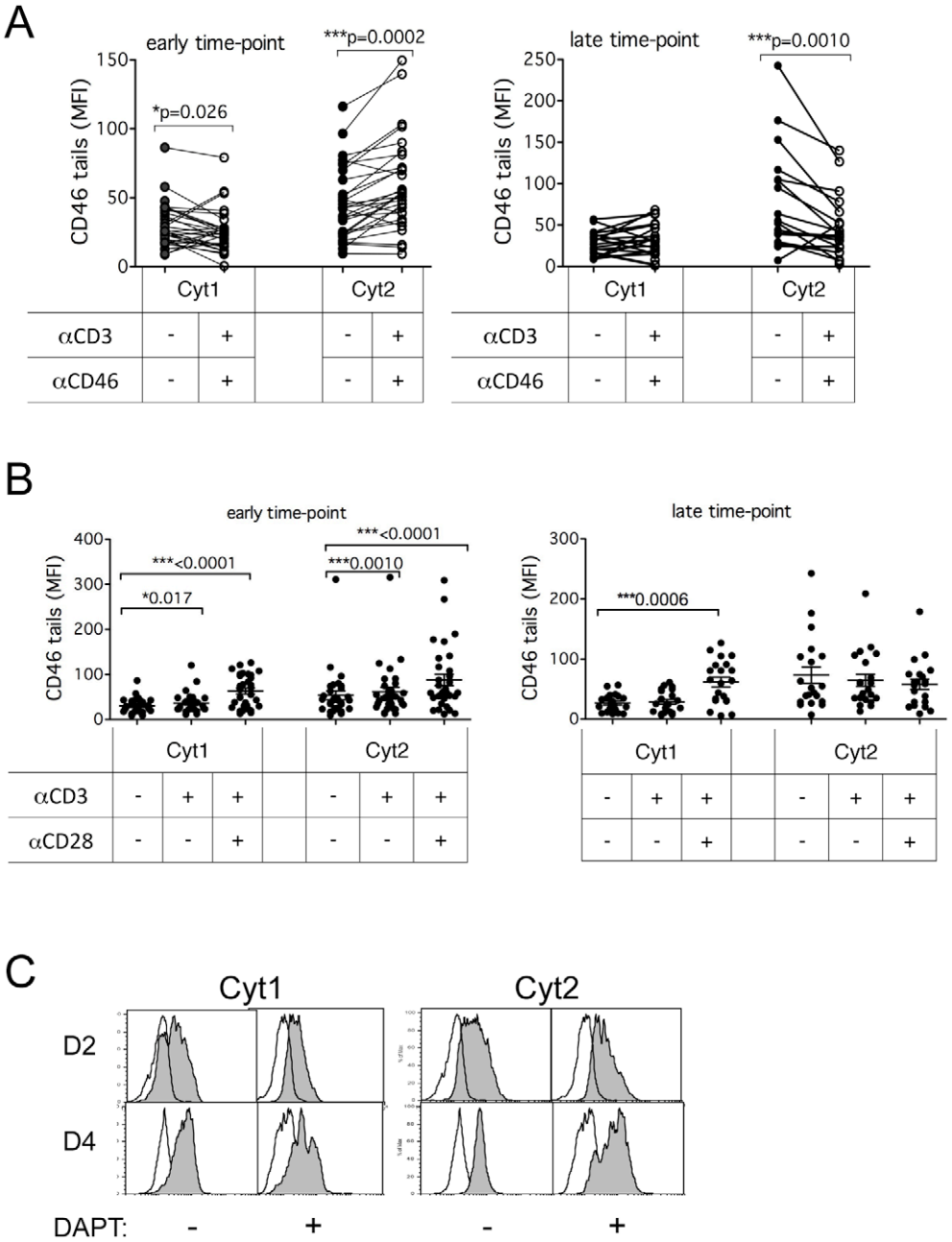


**Figure 2. CD46 cytoplasmic tails are regulated by P/γS upon T cell activation.** Purified $CD4^+$ T cells were left unstimulated or stimulated as indicated by immobilized anti-CD3/CD46 (**A**), anti-CD3 or anti-CD3/CD28 (**B**), for 28–40 hrs (early time point) and 96–120 hrs (late time point). The expression of the two cytoplasmic tails of CD46 was determined by intracellular staining (0.1% saponin) using specific Cyt1 or Cyt2 monoclonal antibodies. The samples were analyzed using the Wilcoxon test, a paired test that does not assume Gaussian variation. The means ± SEM are shown. (**C**) $CD4^+$ T cells were stimulated by immobilized anti-CD3/CD46 antibodies for 2 or 4 days in presence of DAPT (10 μM), a P/γS inhibitor, or of DMSO as control. The expression of Cyt1 and Cyt2 was then analyzed by flow cytometry. There was an increased expression of Cyt1 at D2 and increased Cyt2 levels at D4 in presence of DAPT. Representative of four experiments.
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compared to cells transfected by the empty vector ($p = 0.008$), and slightly decreased the proportion of IFNγ-secreting cells but only upon CD3/CD28 activation. CTF2 expression led to a significant decrease in IFNγ-secreting cells ($p = 0.012$) but had no significant effect on IL-10-secreting cells (Figure 5B,C). CD3/CD46-activated T cells remained mainly insensitive to CTF expression. This is likely due to the dominant stimulation of the endogenous CD46. However, as CTF expression could modulate the response of the cells coactivated by CD28/CD46, this suggests an effect of the CTF on the CD28 pathway, independently of the endogenous CD46, supporting the hypothesis of a crosstalk between CD28 and CD46.

Next, we transfected primary T cells with mutants of CTF1 and CTF2 that cannot be cleaved by P/γS (UNCL.F1 or UNCL.F2, respectively) (Figure 4). A representative experiment from one set of transfected donor cells upon CD3/CD8/CD46 activation is shown in Figure 5C. UNCL.F1-expressing cells secreted ~50% less IL-10 and slightly less IFNγ than CTF1-expressing cells. An increase in

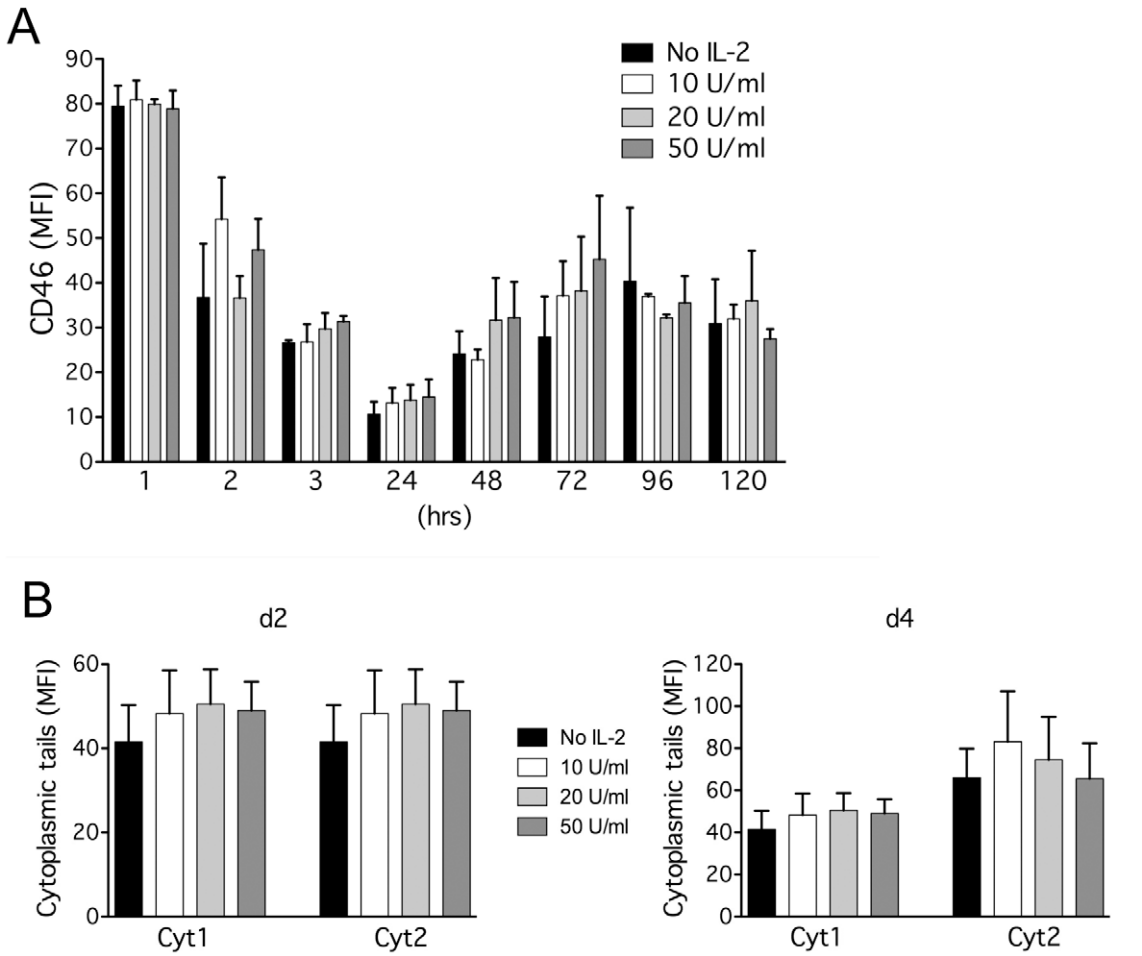


**Figure 3. IL-2 has no significant effect on CD46 expression.** Purified $CD4^+$ T cells were stimulated by immobilized anti-CD3/CD46 with increasing concentrations of rhIL-2, as indicated, for various lengths of time. Expression of cell surface CD46 (**A**) and of intracellular Cyt1 or Cyt2 (**B**) was monitored by flow cytometry. The means ± SEM are shown (n = 5). The samples were analyzed using the Wilcoxon test, a paired test that does not assume Gaussian variation. A slight increase in expression of CD46 cytoplasmic isoforms was observed but no statistical differences were obtained.

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IFNγ production was observed for UNCL.F2-expressing cells compared to CTF2-expressing cells. Although UNCL.F2-expressing cells secreted more IL-10 than CTF2-expressing cells, the proportion of $IL\text{-}10^+$/$IL\text{-}10^+IFN\gamma^+$secreting-cells was unaffected. The proportion of $IL\text{-}10^+$ and $IFN\gamma^+$ only secreting cells upon the different conditions of stimulation obtained for 7 independent donors is also represented in Figure 5D. Inhibition of CTF1 cleavage abrogated the increase in $IL\text{-}10^+$ cells and had a weaker effect on IFNγ-secretion. Inhibition of CTF2 cleavage enhanced the percentage of IFNγ-secreting cells.

We next assessed T cell activation levels by monitoring CD25 expression and proliferation in primary T cells expressing wild-type and uncleavable CTFs. Transfected T cells were labeled with CFSE, then activated with anti-CD3/CD46, anti-CD3/CD28 or anti-CD3/CD28/CD46 antibodies, and CD25 expression and proliferation were assessed by flow cytometry. A representative experiment with one set of transfected donor T cells upon CD3/CD28 activation is presented in Figure 6. CTF1-expressing cells had higher levels of CD25 and proliferated more than control cells. Expression of UNCL.F1 abrogated this effect (Figure 6A). CTF2-expressing cells proliferated similarly than control cells. However, expression of UNCL.F2 led to a strong increase in proliferation rate and CD25 expression, compared to CFT2 expressing cells, as shown by the increased percentage of $CFSEloCD25^+$ cells (inner gate, Figure 6A). The percentage of changes in $CFSEloCD25^+$ cells upon expression of the uncleavable constructs compared to cleavable ones obtained for 7 independent experiments are also represented in Figure 6B. The % of $CFSEloCD25^+$ cells obtained for the different experiments are represented in Figure 6C. UNCL.F1-expressing cells had significant reduced levels of $CFSEloCD25^+$ cells than CTF1-expresing cells, while expression of UNCL.F2 led to a significant increase in $CFSEloCD25^+$ cells. This indicates that processing of the two isoforms regulated cell activation, albeit antagonistically. CTF1 cleavage was necessary to boost T cell activation, while CTF2 cleavage resulted in T cell inhibition.

## Specific triggering of CD46 cytoplasmic tail results in differential T cell activation profile

We next designed fusion constructs that would allow us to directly and specifically trigger Cyt1 or Cyt2 without affecting endogenous CD46. We constructed chimeric molecules consisting of the extracellular domain of CD19, a B cell marker, fused to CTF1 or CTF2 (Figure 7A). These constructs contain the MMP

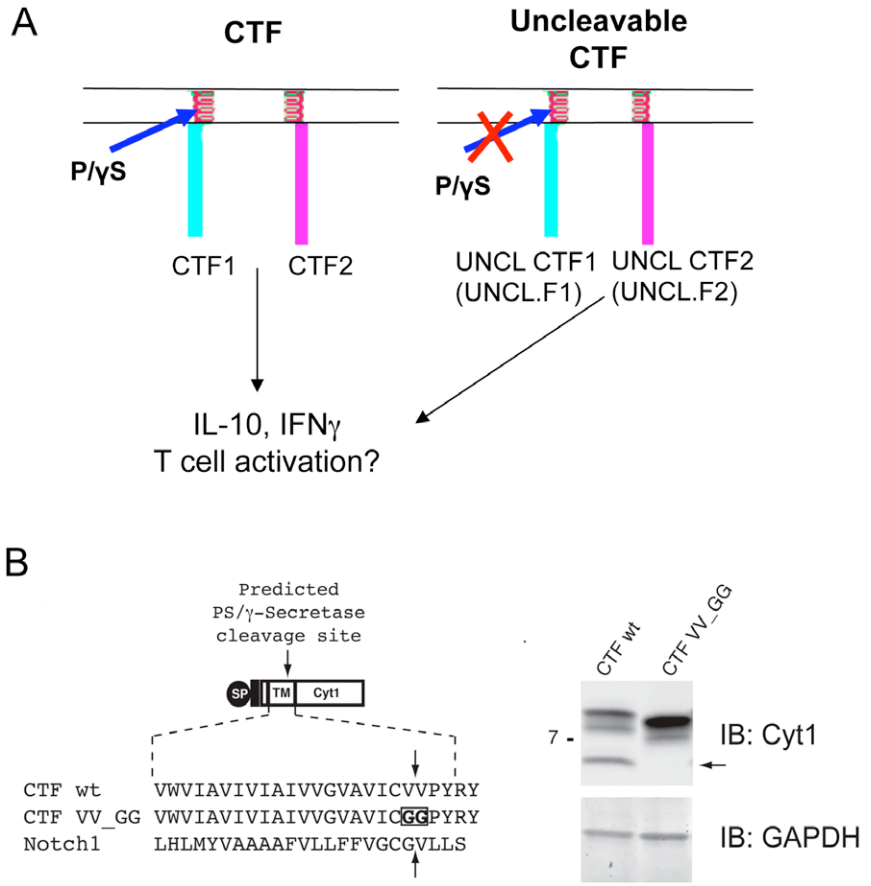


**Figure 4. CTF constructs used in this study.** (**A**) Schematic representation of the proteins encoded by the different CTF plasmids used in this study. Plasmids encoding the CTF portion of either Cyt1 (CTF1) or Cyt2 (CTF2) as well as mutants rendered uncleavable by the P/γS (UNCL.F1 and UNCL.F2) are represented. (**B**) Uncleavable CTF constructs. Left panel: Top diagram shows wt and mutant Cyt1 tail constructs. SP: heterologous signal peptide; black box: peptide linker; unlabelled white box: short segment of CD46 ectodomain; TM: CD46 transmembrane region; Cyt1 (or Cyt2 for CTF2 constructs) cytoplasmic tail. Lower diagram shows amino-acid sequence of the wt TM and uncleavable mutant TMs with amino-acid substitution (boxed residues). The Notch1 TM is shown for comparison. Downward arrow points to predicted P/γS cleavage site for CD46; upward arrow to known cleavage site for Notch1. The right panel shows the immunoblot of CHO cells expressing wt (CTF) and mutant (CTF VV_GG). Arrow indicates the 6 kD Cyt1 peptide release by P/γS cleavage. GAPDH = loading control.
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cleavage domain, and they were named CD19-Cyt1, or CD19-Cyt2. These constructs were first characterized for the correct expression of the chimeric proteins by transfecting them into HEK293 cells and examining fusion protein expression by flow cytometry using antibodies to CD19 ectodomain, Cyt1 and Cyt2 (Figure 7B). Approximately 40% of cells transfected with pcDNA3-CD19-Cyt1 expressed CD19 and high levels of Cyt1 while CD19 and high expression level of Cyt2 was only observed in pcDNA3-CD19-Cyt2 transfected cells. Expression in primary T cells also resulted in ~40% expression of CD19 (Figure 7C). We assessed the functionality of the MMP cleavage site in these fusions. Transfected T cells expressing fusion constructs were left unstimulated, or were activated with immobilized anti-CD3/CD28 antibodies in presence or absence of immobilized anti-CD19 to specifically trigger the fusion proteins, or activated with anti-CD3/CD46, or anti-CD3/CD28/CD46 antibodies. Ligation of CD19 strongly decreased surface levels of CD19 (Figure 7C). This reduced CD19 staining was not due to the masking of the epitope by detached (and pre-immobilized) CD19 antibodies, as the cells did not react with FITC-anti-mouse IgG when harvested from culture (data not shown). These chimeric constructs therefore contain a functional MMP cleavage site.

We next determined whether specific activation of CD19-Cyt1 or CD19-Cyt2 could modulate T cell activation. Because coactivation by CD3/CD28 elicited a much stronger response than CD3 alone (not shown), we subsequently studied the effect of CD19 ligation on CD3/CD28 activation. Primary T cells expressing CD19-Cyt1, CD19-Cyt2 or empty vector were labeled with CFSE and then activated by immobilized anti-CD3/CD46, or by anti-CD3/CD28 antibodies in presence or absence of anti-CD19. After four days, proliferation and CD25 expression were assessed by flow cytometry. A representative experiment is shown in Figure 8A. Expression of CD19-Cyt1 resulted in a decrease of proliferation of the cells compared to cells transfected by the control vector, while CD19-Cyt2 expression by itself, without its ligation, increased proliferation. Ligation of CD19 had no significant effect of cells transfected by the control or expressing CD19-Cyt1. In contrast, a dramatic inhibition of proliferation was observed when CD19-Cyt2 was engaged. While no significant difference in CD25 expression was detected for the control cells and CD19-Cyt1 expressing cells, a strong decrease in CD25 expression was observed for CD19-Cyt2 expressing cells upon CD19 ligation (Figure 8A and B). These results therefore supported those observed using the CTF constructs. Expression of CD19-Cyt1 mainly mimicked UNCL.F1

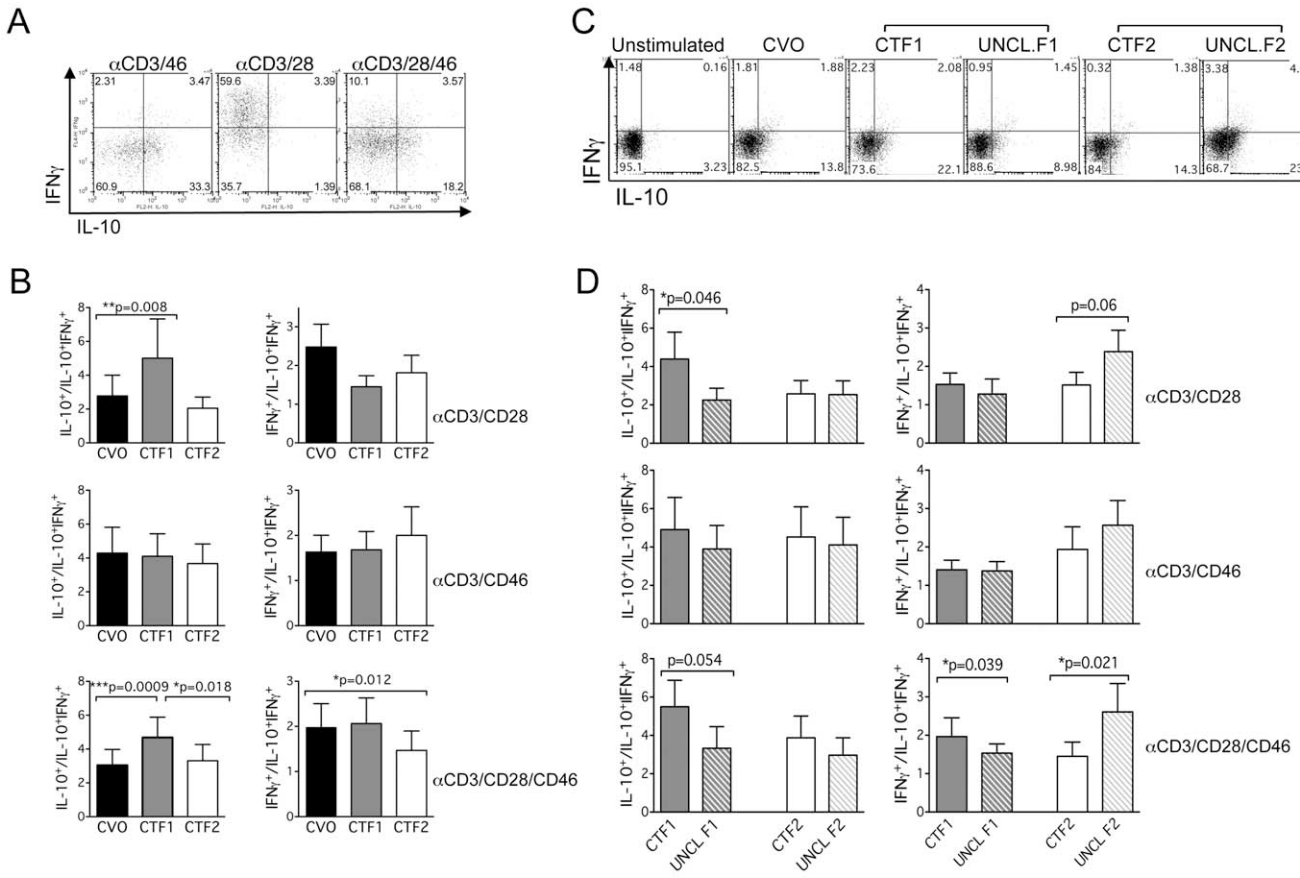


**Figure 5. Cleavage of CTF-Cyt1 and CTF-Cyt2 differently controls IL-10 and IFNγ production.** (**A**) Activation of transfected (with a control plasmid) primary $CD4^+$ T cells results in normal differentiation. Twenty-four hours post transfection by Amaxa/Lonza with the empty vector control (CVO), $CD4^+$ T cells were stimulated by immobilized anti-CD3/CD46, anti-CD3/CD28 or anti-CD3/CD28/CD46 monoclonal antibodies for 72 hrs. The secretions of IL-10 and IFNγ were assessed by secretion assays (Miltenyi). CD46 costimulation induces mainly IL-10 production, while CD28 coactivation induces more IFNγ. CD28/CD46 coligation also induces Tr1/IL-10 secretion (as described by [15]). (**B**) $CD4^+$ T cells were transfected with the control plasmid (CVO) or encoding CTF-Cyt1 (CTF1) or CTF-Cyt2 (CTF2) fragments. Twenty-four hours post transfection, $CD4^+$ T cells were stimulated by immobilized anti-CD3/CD28, anti-CD3/CD46 or anti-CD3/CD28/CD46 monoclonal antibodies, as indicated. After 3 days, the secretions of IL-10 and IFNγ were assessed by secretion assays (Miltenyi). The proportions of $IL\text{-}10^+/IL\text{-}10^+IFN\gamma^+$ and of $IFN\gamma^+/IL\text{-}10^+IFN\gamma^+$ secreting cells for the multiple experiments performed are represented. The means ± SEM are shown (n = 10). All data were analyzed using the Wilcoxon test, a non-parametric paired t-test that does not assume Gaussian distribution. (**C**) $CD4^+$ T cells were transfected with the plasmids encoding CTF-Cyt1 (CTF1) or CTF-Cyt2 (CTF2) or with the uncleavable CTF1 or CTF2 (UNCL.F1 and UNCL.F2) before analysis of IL-10 and IFNγ production. The data obtained for one donor is shown upon CD3/CD28/CD46 activation. (**D**) The proportions of $IL\text{-}10^+/IL\text{-}10^+IFN\gamma^+$ and of $IFN\gamma^+/IL\text{-}10^+IFN\gamma^+$ secreting cells for the multiple experiments performed are represented (n = 7).
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expression while CD19-Cyt2 expression mainly reflects UNCL.F2 expression. However, specific activation of CD19-Cyt2 led to a striking inhibition of proliferation and CD25 expression, and indicated that Cyt2 differently modulates T cell activation depending on its triggering and processing.

We next determined the effect of Cyt1 and Cyt2 specific triggering on cytokine production. T cells expressing CD19-Cyt1 and CD19-Cyt2 were activated by CD3/CD28 or CD3/CD28/CD19, and cytokine production determined. The percentage of change in cytokine production upon CD19 ligation was calculated for $IL\text{-}10^+$, $IL\text{-}10^+IFN\gamma^+$ and $IFN\gamma^+$ cells. Co-activation of CD19-Cyt1 cells increased the percentage of cells producing IL-10 only ($p = 0.019$; Figure 8C). CD19-Cyt1 had no significant effect on the proportion of $IL\text{-}10^+IFN\gamma^+$ cells and on $IFN\gamma^+$ secreting cells. In contrast, CD19 co-activation of CD19-Cyt2 cells significantly reduced IFNγ production ($p = 0.037$). Finally, CD19 co-activation of vector only control cells had no effect on either IL-10 or IFNγ production. These results corroborate the findings from CTF-expressing cells that altered the proportion of $IL\text{-}10^+$ or $IFN\gamma^+$-only producing cells.

## Specific triggering of CD19-Cyt2 results in increased CTLA-4 expression and dephosphorylation of LAT

In order to determine a possible mechanism for the inhibitory effect of Cyt2, we analyzed CTLA-4 levels, a potent co-inhibitory molecule for T cells [30]. As shown in Figure 9A, CTLA-4 expression is induced by activation of primary human T cells, and notably when CD46 is stimulated. In transfected cells, activation by CD19 had no effect on CD19-Cyt1 expressing cells and control cells. However, triggering of CD19-Cyt2 led to a strong increase in CTLA-4 expression, providing a possible mechanism for Cyt2 inhibitory role. The results obtained for one experiment is shown in Figure 9B, and the average data obtained for the different donors represented in Figure 9C.

CTLA-4 engagement decreases T cell activation by transmitting negative signals. Notably, it induces the dephosphorylation of

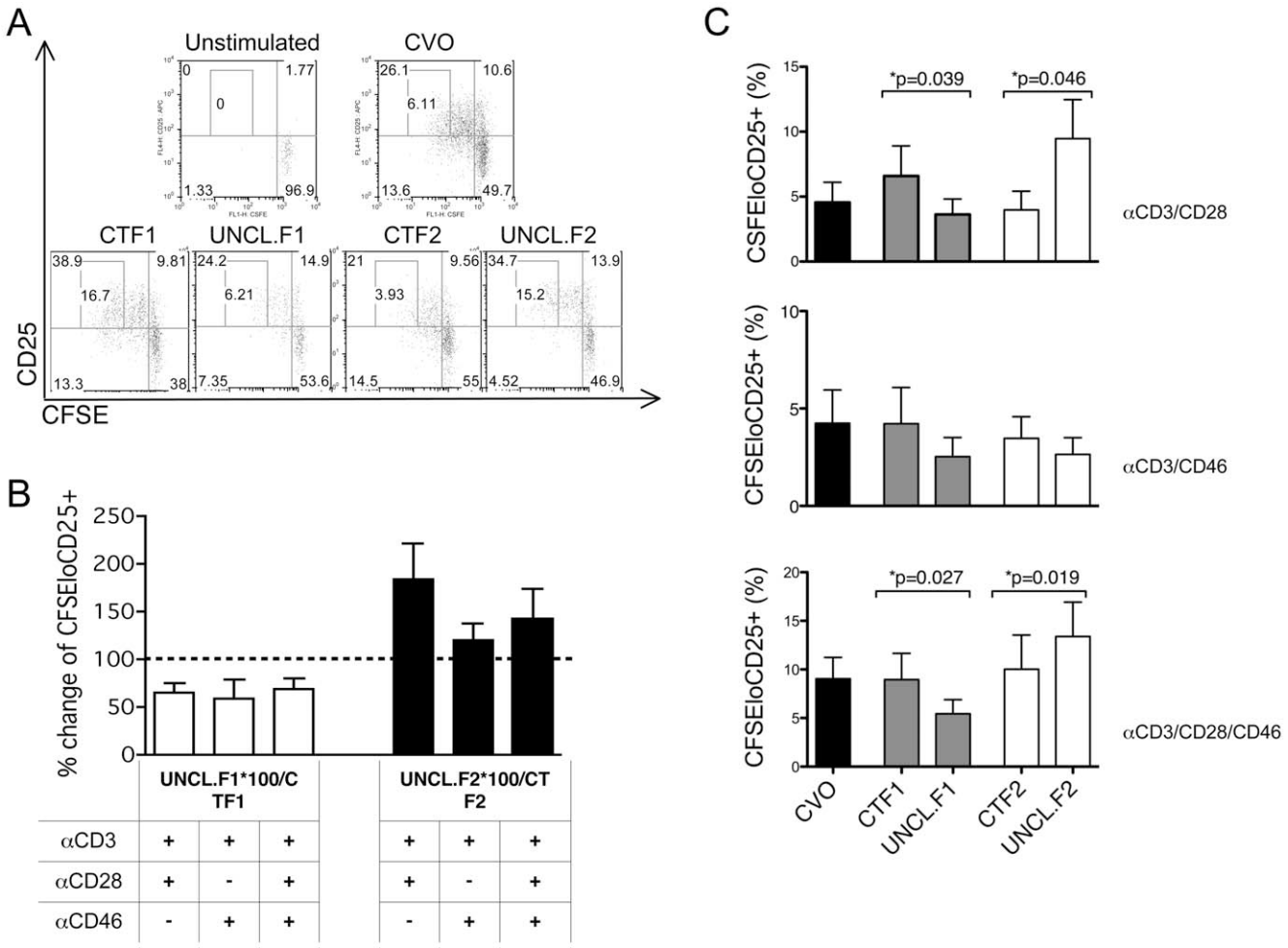


**Figure 6. Cleavage of CTF-Cyt1 and CTF-Cyt2 differently controls T cell proliferation and CD25 expression.** $CD4^+$ T cells were transfected with the plasmid encoding CTF-Cyt1 (CTF1) or CTF-Cyt2 (CTF2), or the uncleavable fragments (UNCL.F1and UNCL.F2). Twenty-four hours post transfection, $CD4^+$ T cells were labeled with CFSE and stimulated with immobilized antibodies for 4 days. Proliferation and CD25 expression was determined by flow cytometry. (**A**) The data obtained upon CD3/CD28 activation for one donor is shown. (**B**) The percentage of change in $CFSEloCD25^+$ cells upon expression of the uncleavable constructs compared to the cleavable ones upon the different conditions of stimulation is represented as an average of several independent experiments with different donors (n = 7). (**C**) The average % of $CFSEloCD25^+$ cells (inner gate in (**A**)) obtained in the different experiments performed is also represented.
doi:10.1371/journal.pone.0016287.g006

proteins activated by TCR stimulation, such as LAT [31]. CD46 activation of T cells induces LAT phosphorylation [12]. Hence, we next determined the level of LAT phosphorylation upon activation of each tail. $CD4^+$ T cells were transfected with the control plasmid or the plasmids encoding CD19-Cyt1 or CD19-Cyt2 and activated for 5 min in presence of anti-CD3/CD28 with or without CD19 ligation. The phosphorylation of LAT was analyzed by flow cytometry. The results obtained for one experiment are shown in Figure 9D, and the average data obtained for the different donors represented in Figure 9E. As expected, no difference was observed for the control cells when CD19 was activated. No significant effect was observed by the simultaneous triggering of CD19-Cyt1 and CD3/CD28. In contrast, CD19-Cyt2 costimulation induced a significant decrease in LAT phosphorylation, supporting the hypothesis of the role of Cyt2 in terminating T cell activation.

## Discussion

The outcome of T cell activation results from a computation of signals received by the T cells, ensuring first proper activation, followed by adequate termination of the immune response initiated [1]. Here, we demonstrate the ability of CD46, a major costimulatory molecule for human T cell activation [12], to provide both coactivation and termination signals, through the regulation of expression of its two cytoplasmic isoforms. We first demonstrate the regulation of CD46 processing. An MMP-dependent cleavage of CD46 ectodomain was initially observed at the cell surface of T cells when CD46 was ligated. The antibodies used for activation and labeling were different, and there was a clear effect of MMP inhibitor indicating that an MMP-dependent cleavage was at least partly involved. We also investigated whether CD46 activation induced its internalization by measuring intracellular levels of CD46 after intracellular staining and acid-stripping of the cells, as reported in [32] (data not shown). No significant increase in CD46 was detected in these conditions, suggesting that most CD46 was shed from the surface. This also correlated with the presence of soluble CD46 in the culture supernatants of coactivated T cells. Apoptosis of neuronal and epithelial cells also triggers CD46 MMP-dependent shedding [33,34], indicating that several biological pathways use this

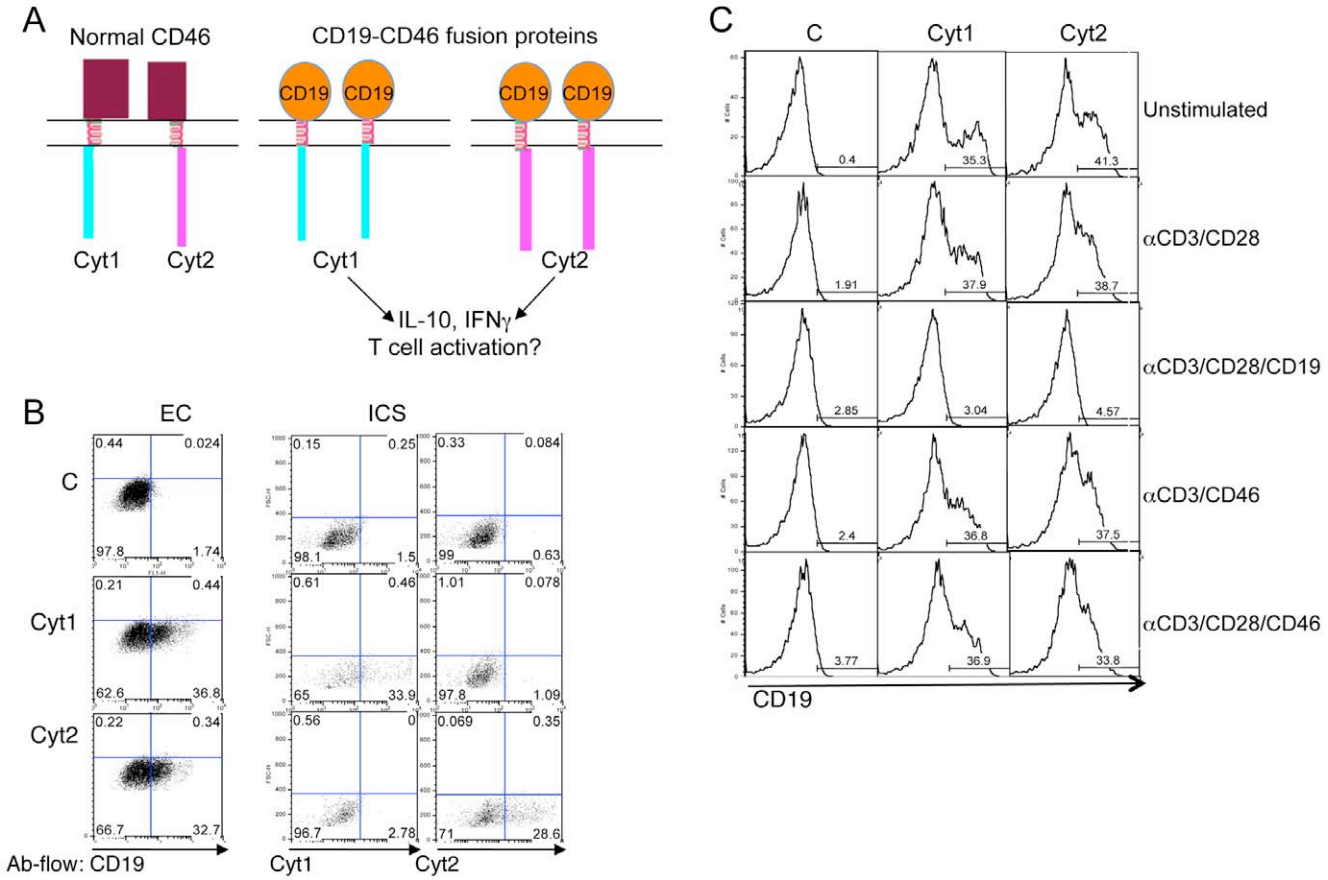


**Figure 7. CD19-CTF fusion proteins used in this study.** (**A**) Schematic representation of the proteins encoded by the different fusion plasmids used in this study. Plasmids encoding the CD19 ectodomain fused to either CTF-Cyt1 or CTF-Cyt2 was constructed. (**B**) Chimeric protein expression in HEK293 cells. HEK cells were transfected by Fugene with pcDNA3 plasmids (control (C), CD19-Cyt1 (Cyt1) or CD19-Cyt2 (Cyt2)). Twenty-four hours later, the expression of CD19, Cyt1 and Cyt2 was assessed by flow cytometry using anti-CD19-FITC and monoclonal antibodies specific of each isoform. (**C**) $CD4^+$ T cells were transfected with the control pcDNA3 plasmid (C) or encoding the fusion proteins consisting of the extracellular domain of CD19 and CTF of either Cyt1 or Cyt2 and activated as indicated. Twenty-four hours later, CD19 expression was determined by flow cytometry. Representative of three experiments.
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mechanism of regulation. The loss of CD46 expression at the cell surface was followed by the downregulation of its cytoplasmic isoforms, although with a strikingly different time-course. Cyt1 was downregulated first, while Cyt2 downregulation was observed afterwards. The decrease in Cyt1 and Cyt2 expression could be partially restored in presence of P/γS inhibitors, in a timely fashion. This suggests that, upon T cell activation, this enzymatic complex cleaves at least a part of CD46 cytoplasmic tails. We were barely able to detect by western-blots these fragments in activated primary T cells (not shown). This may be due to the short half-life of these fragments once released in the cytosol and the limit in sensitivity of our assay with primary T cells. However, we could detect the decrease in full length CD46 upon its activation (not shown). Importantly, the processing by P/γS of both CD46 cytoplasmic isoforms has been observed upon binding of the pathogenic *Neisseria* bacteria, *N. gonorrhoeae* and *N. meningitidis*, to epithelial cells [28]. Hence, CD46 processing might be a general pathway initiated by its triggering, producing active ICD that will transduce signals.

Most importantly, we demonstrate herein the dual role of CD46 in regulating human T cell activation. Ten years ago, it was discovered that CD46 could act as a costimulatory molecule for human T cells [12] and few years later that it could drive Tr1 differentiation [15]. Our data now illustrate the role of CD46 in turning off T cell activation, providing a novel concept in the regulation of the immune response. This "yin and yang" ability of CD46 in regulating T cell activation is mediated by the P/γS-dependent processing of its two cytoplasmic isoforms. Activation of T cells by CD46 leads to Tr1 differentiation [15]. Our data indicate that Tr1 differentiation initiated by CD46 activation is mainly due to Cyt1, as its specific activation using the CD19-CD46 fusion protein promoted IL-10 secretion, previously characterized as a marker of CD46-induced Tr1 cell differentiation [15,16]. An increase in Granzyme B, another hallmark of CD46-induced Tr1 cells [16] was also observed (data not shown). Importantly, the role of Cyt1 in IL-10 production was corroborated by the CTF constructs, as expression of CTF1 increased the proportion of IL-10-secreting cells. It also promoted T cell proliferation and CD25 expression. The cleavage of CTF1 was required for these functions, as they were abrogated by expression of the uncleavable CTF1 mutant. As CD3/CD28 activation induced a much stronger response than CD3 activation alone, we mainly studied the effects of CTF on CD3/CD28 or CD46 co-activated T cells. However, we were able to detect similar effects of the CTF constructs on CD3-activated T cells when CD3 induced sufficient activation levels (Figure S4). Expression of uncleavable

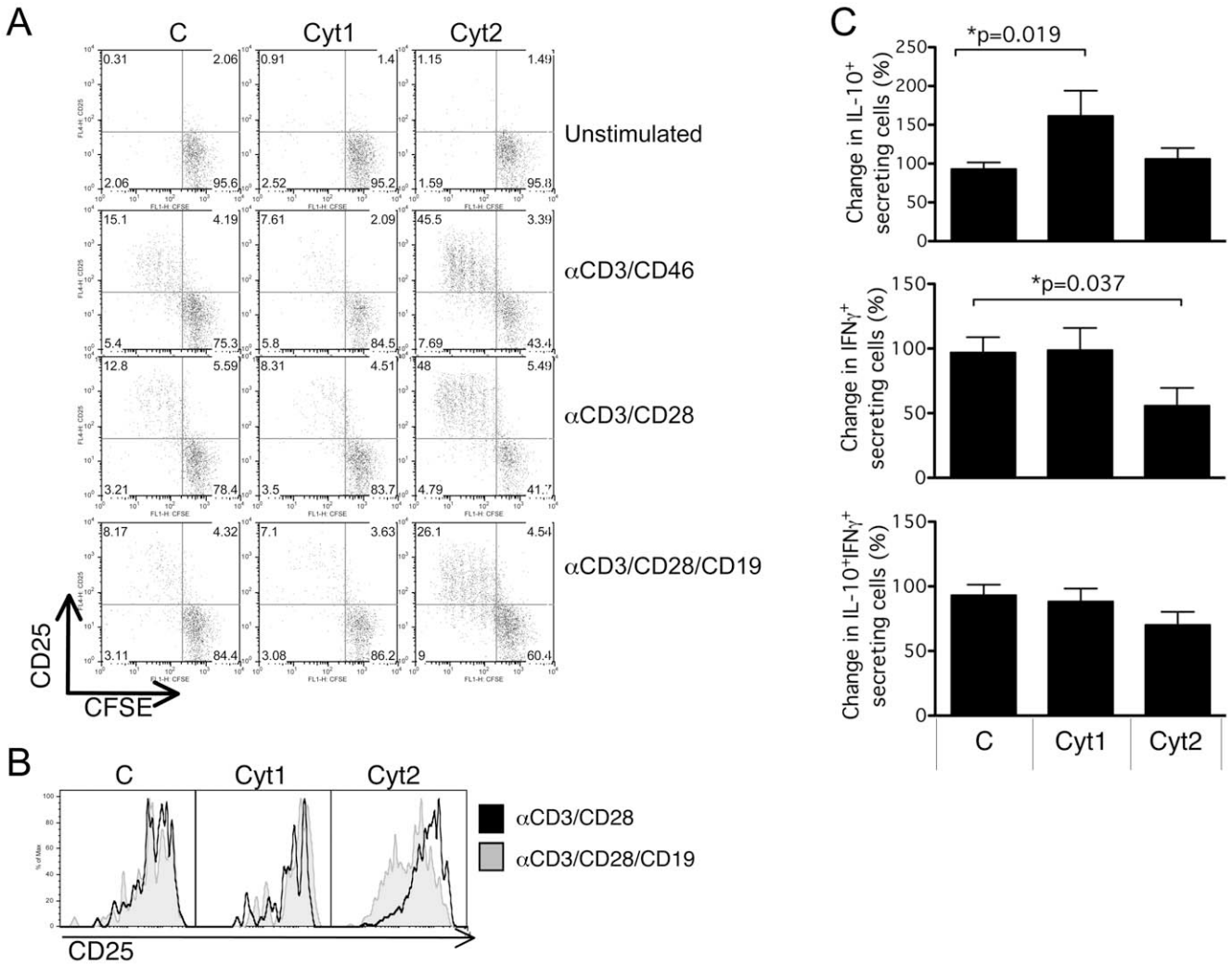


**Figure 8. Specific triggering of Cyt1 and Cyt2 differently controls T cell activation.** (**A**) $CD4^+$ T cells were transfected with the control pcDNA3 plasmid (C) or encoding the CD19-CTF1 (Cyt1) or CD19-CTF2 (Cyt2) fusion proteins. Twenty-four hours post transfection, $CD4^+$ T cells were labeled with CFSE. Labeled cells were then stimulated by immobilized anti-CD3/CD46, anti-CD3/CD28 or anti-CD3/CD28/CD19 monoclonal antibodies and proliferation as well as CD25 expression were determined by flow cytometry. (**B**) Proliferating cells were gated on CFSE-low cells and the expression of CD25 was determined. The black lines represent the staining obtained for CD3/CD28 activated cells, the shaded grey histograms represent the staining for the cells activated in presence of anti-CD19. Representative of three experiments. (**C**) Transfected cells were activated by anti-CD3/CD28 or anti-CD3/CD28/CD19 monoclonal antibodies. After 4 days, the secretions of IL-10 and IFNγ were assessed by secretion assays (Miltenyi). The changes in the percentages of $IL\text{-}10^+$, $IL\text{-}10^+IFN\gamma^+$ and $IFN\gamma^+$ secreting cells induced by CD19 ligation compared to secretion in absence of CD19 (100%) for the multiple experiments performed are represented (n = 9). Samples were analyzed using the Wilcoxon test.
doi:10.1371/journal.pone.0016287.g008

CTF1 appears to block the cells in an unactivated state and fewer cells get activated. Expression of CD19-Cyt1 has the same effects – there are fewer cells capable of being activated. Hence, the decrease in proliferation of CD19-Cyt1 expressing cells, without its ligation, correlates with the results obtained by expressing UNCL.F1. The increase in proliferation and CD25 expression was not detected in CD19-activated cells expressing CD19-Cyt1 fusion protein. This may relate to the strength of activation of the cells, as the effect of CTF1 was only observed in CD3/CD28 co-activated T cells. The specific engagement of Cyt2 led to strikingly different effects. Expression of uncleavable CTF2 strongly promotes T cell activation. Similar results are obtained when CD19-Cyt2 is expressed. This suggests that Cyt2 can trigger signaling events independently of extracellular ligation, and participates in T cell activation at the early time point. Specific Cyt2 activation, via CD19 ligation, provokes its cleavage as demonstrated by the loss of CD19 expression upon its engagement, and results in lowered IFNγ secretion, as well as dramatically decreased proliferation and CD25 expression compared to CD3/CD28 activation in absence of CD19. Similarly, expression of an uncleavable CTF2 resulted in an increased IFNγ production, enhanced proliferation and CD25 levels. Furthermore, specific triggering of Cyt2 could enhance CTLA-4 level, a potent co-inhibitory molecule for T cells [30], providing a possible mechanism for Cyt2 inhibitory role. Indeed, we could demonstrate that specific Cyt2 triggering led to a decrease in LAT phosphorylation. Interestingly, CD46-activated T cells do not sustain proliferation over longer activation periods [35]. We have shown that gated $CTLA\text{-}4^+$ cells exhibit a strong inhibition of proliferation when Cyt2 in engaged (Figure S5). Our data suggest an involvement of Cyt2 by upregulation of CTLA-4 and subsequent dephosphorylation. Together, these data and the

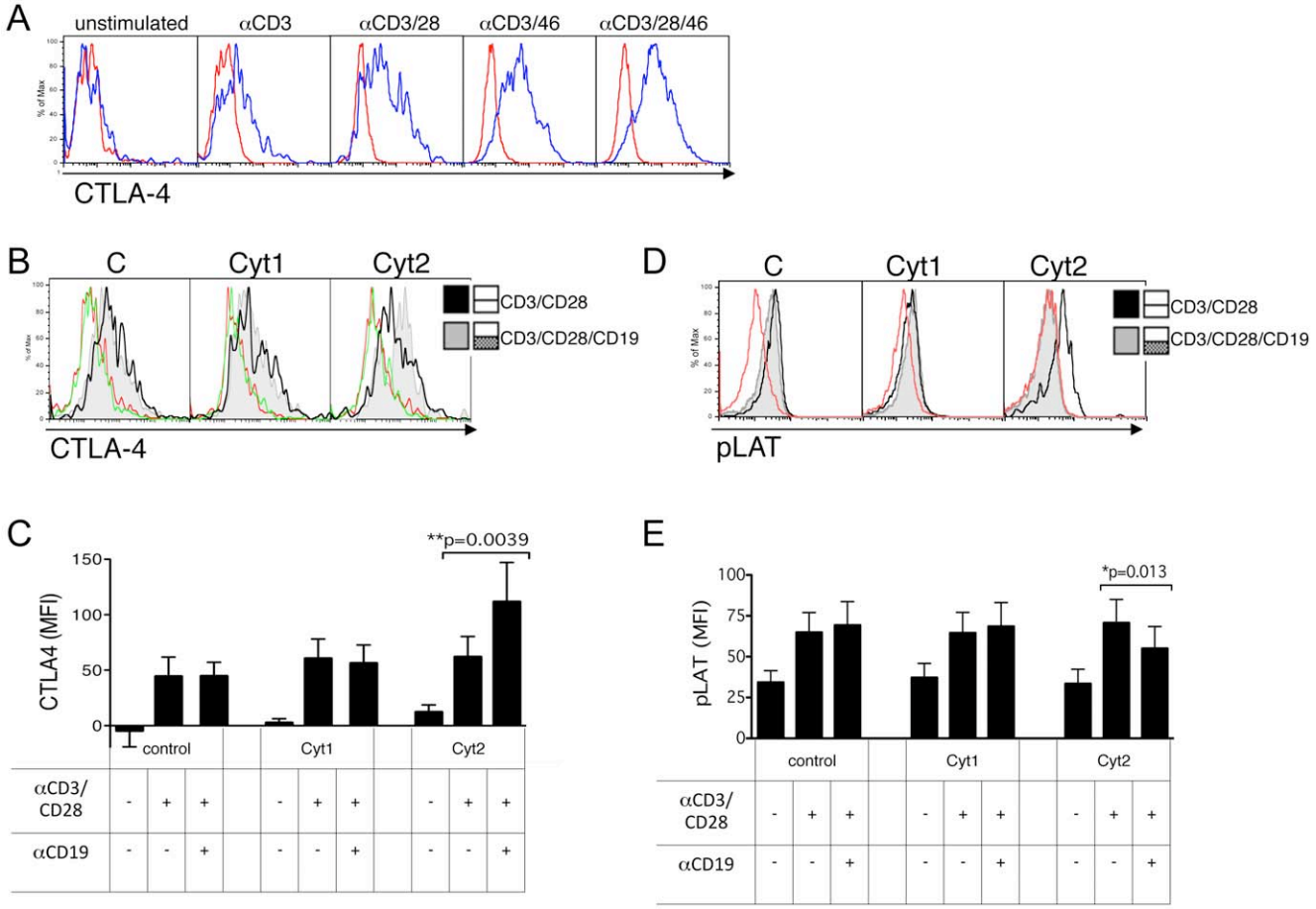


**Figure 9. Specific Cyt2 triggering increases CTLA-4 expression and decreases LAT phosphorylation.** (**A**) CTLA-4 expression was determined by flow cytometry on untransfected primary T cells activated as indicated for 4 days. (**B**) Twenty-four hours post transfection with pcDNA3 plasmids (control, CD19-Cyt1 or CD19-Cyt2), $CD4^+$ T cells were stimulated with anti-CD3/CD28 (black plain line) or anti-CD3/CD28/CD19 (shaded grey area) monoclonal antibodies for 5 days. The expression of intracellular CTLA-4 was then assessed by flow cytometry. Red and green lines represent the staining obtained with the isotype controls for both types of activation. The average normalized CTLA-4 staining obtained for 5 experiments is represented in (**C**). Transfected cells were activated with anti-CD3/CD28 with or without CD19 and addition of a cross-linker for 5 min at 37°C. Activated cells were immediately fixed and then permeabilized. The presence of phospho-LAT was detected with an anti-pLAT by flow cytometry. Data representative of one donor are shown in (**D**). Red lines represent the staining obtained with the isotype controls. The average normalized pLAT staining obtained for 4 experiments is represented in (**E**).
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results obtained using CTF2 clearly demonstrate that the lack of cleavage results in increased T cell activation. This illustrates that CTF2 processing is required to trigger inhibitory signals within activated T cells, in a negative feedback mechanism.

Importantly, while this manuscript was being written, it was reported that CD46 activation could switch T cells from a Th1 toward to Tr1 phenotype depending on IL-2 concentrations present in the milieu [17]. However, in our hands, we did not observe significant effects of increasing IL-2 concentrations on CD46 processing. Remarkably though, the authors found that expression of Cyt1 in Jurkat cells led to increased IL-10 secretion. Moreover, ligation of CD46 in γδ T cells that express more Cyt2 than Cyt1 (at least by PCR) results in a decrease in IFNγ expression. Our data corroborate these findings in primary $CD4^+$ T cells, but they also demonstrate the role of CD46 isoforms in overall primary human T cell activation, and we provide evidence of the requirement of their processing in such functions. Importantly, both studies highlight the importance of the plasticity of CD46 in controlling T cell activation. While Kemper's group shows the ability of CD46 to switch cytokine production depending on IL-2 concentrations, we demonstrate that within the Tr1 differentiation conditions, CD46 acts as a rheostat for T cell activation. We first observed the loss of expression of Cyt1, while Cyt2 processing occurred later. This supports the notion that the initial regulation of CD46-Cyt1 induces IL-10 production and Tr1 differentiation, while the later Cyt2 processing results in switching off Tr1 cells, in a negative feedback mechanism. We had previously reported that CD46 cytoplasmic isoforms had differential roles in inflammation using transgenic mice expressing either isoform. In an *in vivo* model of contact hypersensitivity reaction to DNFB, CD46-Cyt1 had a potent anti-inflammatory role, while Cyt2 promoted inflammation [27]. Our data in human cells corroborate the anti-inflammatory role of Cyt1 through activation and Tr1 differentiation, and the pro-inflammatory role of Cyt2 may be explained by decreased IFNγ production [36,37] and lack of Tr1 differentiation. Importantly, CD46 is defective in MS, as CD46-activation of both T cells and dendritic cells leads to a pro-inflammatory phenotype [19,38]. Whilst CD46 activation induced Tr1 differentiation and IL-10 secretion in T cells from healthy donors, IL-10 production was decreased by T cells from

MS patients [19,20,21,39]. This was associated with an increased RNA expression of the Cyt2 isoform upon T cell activation [19]. Our data now reinforce the likely role of the abnormal Cyt1/Cyt2 ratio in the lack of Tr1 differentiation in patients with MS.

Overall, our data suggest that CD46, through the temporal processing of its cytoplasmic tails, acts as a molecular rheostat for human T cells. We propose that, first, Cyt1 is cleaved which promotes T cell activation and IL-10 production, and that, later, Cyt2 cleavage sends a negative feedback message, turning off T cell activation, as summarized in the model depicted in Figure 10 and based on the data presented in Figure S6. Further elucidation of the transduction cascades initiated by CD46 isoforms will be required to fully understand their role in human T cell activation. The understanding of the mechanisms regulating their expression will provide a potent tool for immuno-therapies and will complete our growing knowledge on T cell activation and contraction in health and disease.

## Materials and Methods

### Cell purification and activation

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ), from heparinized venous blood from healthy donors obtained after informed consent. Ethical approval was obtained from the Lothian Board Ethics Committee. $CD4^+$ T cells were negatively isolated using magnetic beads (CD4 isolation kit II, Miltenyi Biotec, Auburn, CA, purification >90%). T cells were then cultured in culture wells pre-coated with anti-CD3 (OKT3, 5 μg/ml), anti-CD28 (CD28.2, 10 μg/ml), anti-CD46 (10 μg/ml) (20.6, kindly provided by Dr. Chantal Rabourdin-Combe, France), anti-CD19 (AbD Serotec, 10 μg/ml), or irrelevant IgG1 (Invitrogen, 10 μg/ml). Exogenous IL-2 (10 U/ml) was added to CD3/CD46 stimulated cells as previously described [15]. In some experiments, the P/γS inhibitors DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) or L-685,458 (Sigma-Aldrich) were added to the culture.

### Cell transfection

$CD4^+$ T cells were negatively isolated (Miltenyi, purification >90%) and transfected (Amaxa/Lonza), following the manufacturer's instructions (U-14). HEK293 cells were transfected by Fugene (Roche). Twenty-four hours later, the expression of CD19, Cyt1 and Cyt2 was assessed by flow cytometry using CD19-PE (BD Biosciences), or specific monoclonal antibodies against Cyt1 or Cyt2 [29] (in 0.1% saponin).

### CTF constructs

The CTF expression constructs were made by subcloning PCR products amplified from CD46 pSecTag2/Hygro (Invitrogen) clones for Cyt1 and Cyt2 into pIRESneo (GenBank Accession no. U89673). PCR products were generated using primers CTF_IRES_EcoR1_F with IRES_BamHI_R_CTF1 for Cyt1 and CTF_IRES_EcoR1_F and IRES_BamHI_R_CTF2 for Cyt2. PCR products were digested with EcoR1 and BamH1 prior to cloning into pIRESneo. The predicted P/γS cleavage site was mutagenized with the VV_GG and VV_GG_rc primers using the QuikChange Lightning Site-Directed mutagenesis kit (Stratagene) according to the manufacturers instructions (Figure 4).

### Fusion proteins

The extracellular domain of CD19 was fused to CD46 CTF1 or CTF2. After total RNA extraction from HeLa and Raji cells, cDNAs were obtained using specific primers: AA3 for CD19, AA4 for Cyt-1 and AA5 for Cyt-2 (Table 1). The CD19 extracellular region was amplified by PCR with the primers AA1 and AA2 using PHUSION hot start polymerase (Finnzyme) with the addition of *HindIII* and *XbaI* restriction sites at the 5′ and 3′ ends, respectively. Cyt1 and Cyt2 transmembrane and intracytoplasmic domains were amplified with the pairs of primers AA3, AA4 and AA3, AA5 respectively. Amplified CD19, Cyt1 and Cyt2 cDNA were cloned in pBluescript II plasmid (Stratagene) and sequenced. Chimeric molecules containing CD19 extracellular region fused to the CTF domain of either CD46-Cyt1 or Cyt2 isoform were created by SOE-PCR [40,41] using PHUSION hot start polymerase. For chimeric CD19-Cyt1, primer AA1 annealed at the 5′ end of CD19; primer AA4 annealed at the 3′ end of Cyt1 cDNA. Primers AA6 and AA7 were complementary to each other, overlapping at the respective fusion point of the two cDNA molecules. The chimeric cDNA was cloned into pcDNA3 vector

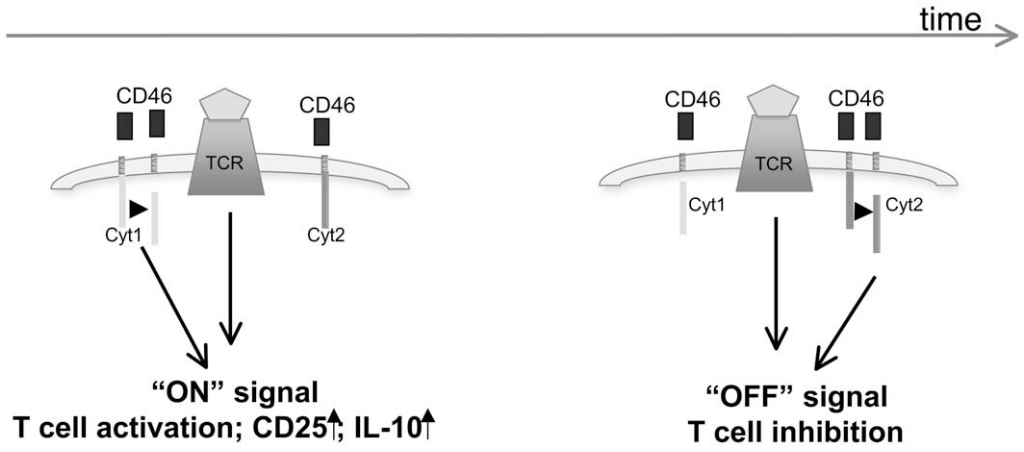


**Figure 10. Proposed model of the yin and yang role of CD46 in human T cell activation.** Upon CD3/CD46 activation, there is downregulation of Cyt1 expression and increase in Cyt2 expression at the early time point compared to unstimulated T cells. Cyt1 expression level then returns to the level of expression observed in unstimulated T cells, while the downregulation of Cyt2 expression occurs at the late time point. We propose that Cyt1 isoform is first cleaved by P/γS while there is an increase in Cyt2 expression. This results in T cell activation, increased proliferation and CD25 expression as well as IL-10 secretion. Later on, Cyt2 is processed by the P/γS, which induces a negative feedback mechanism and results in T cell inhibition, with lowered IFNγ secretion, decreased proliferation and CD25 expression and increased CTLA-4 expression.
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**Table 1.** Primers used for cDNA cloning and generation of CTF and chimeric CD19-CD46 molecules[1].

| PRIMER NAME | SEQUENCE |
|---|---|
| **CTF CONSTRUCTS** | |
| CTF_IRES_EcoR1_F | CTAGAATTCCCACTGCTTACTGGCTTATCG |
| IRES_BamHI_R_CTF1 | GATCGGATCCTCAGAGAGAAGTAAATTTTACTTCTCTGTGG |
| RES_BamHI_R_CTF2 | GATCGGATCCTCAGCCTCTCTGCTCTGCTGGAG |
| VV_GG | GTTGGAGTTGCAGTAATTTGTGGTGGCCCGTACAGATATCTTCAAAG |
| VV_GG_rc | CTTTGAAGATATCTGTACGGGCCACCACAAATTACTGCAACTCCAAC |
| **FUSION PROTEINS** | |
| AA1 | 5′-CCCCCC**AAGCTT**AGTCTGACCACCATGCCACC-3′ |
| AA2 | 5′-CCCCCC**TCTAGA**CTTCCAGCCACCAGTCCTCAG-3′ |
| AA3 | 5′-CCCCCC**AAGCTT**GATGTTTGGGTCATTGCTGTG-3′ |
| AA4 | 5′- CCCCCC**TCTAGA**TCAGAGAGAAGTAAATTTTACTTCTC-3′ |
| AA5 | 5′-CCCCCC**TCTAGA**TCAGCCTCTCTGCTCTGCTG-3′ |
| AA6 | 5′-CAGCAATGACCCAAACATCCTTCCAGCCACCAGTCCT-3′ |
| AA7 | 5′-AGGACTGGTGGCTGGAAGGATGTTTGGGTCATTGCTG-3′ |

[1]Bold letters indicate HindII and XbaI restriction sites. Overlapping sequences for generation of chimeric cDNA are underlined.
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(Invitrogen) using the *HindIII* and *XbaI* restriction sites introduced in the cDNA. The chimeric CD19-Cyt2 construct was similarly generated, using primers AA1, AA5, AA6 and AA7. All constructs were verified by sequencing.

### Proliferation assay

Eighteen hours post transfection, T cells were labeled with CFSE after extensive washes with cold PBS, for 10 min at 37C. After quenching the reaction with cold 10%RPMI and further washes, the cells were seeded in 96-well plates precoated with immobilized antibodies as indicated. Four days later, the proliferation and CD25 expression (anti-CD25-APC) was assessed by flow cytometry.

### IFNγ and IL-10 secretion assays

Eighteen hours post transfection, T cells were seeded into 48-well culture plates ($2.5\times10^5$ cells per well) pre-coated with various antibodies. Four days later, the cells were harvested and the amounts of IL-10- and IFNγ-secreting cells were determined using the secretion assays from Miltenyi (IL-10-PE; IFNγ-APC).

### Flow cytometry

The expression level of CD46 ectodomain was assessed by flow cytometry with anti-CD46-FITC (BD Pharmingen). The expression level of Cyt1 or Cyt2 was performed by intracellular flow cytometry staining (with 0.1% saponin) using the specific monoclonal anti-Cyt1 or Cyt2 antibodies previously generated [29]. The relative expression to the staining with the isotype control was calculated by calculating the ΔMFI (MFI antibody stained - MFI control antibody).

### Detection of phospho-LAT

Transfected cells were activated with the appropriate Abs (CD3/CD28 with CD19 or irrelevant IgG1) and addition of a cross-linker for 5 min at 37°C [12]. Activated cells were immediately fixed (Cytofix, BD Biosciences) and then permeabilized (Perm buffer, BD Biosciences). The presence of phospho-LAT was detected with an anti-pLAT (pY171; BD Biosciences) and analyzed by flow cytometry.

### Statistical analyses

The groups were analyzed using the Graphpad Prism software. Data were analyzed using the Wilcoxon test, a non-parametric test that does not assume Gaussian variation. All p-values are two-tails and with a 95% confidence interval.

## Supporting Information

**Figure S1 Addition of the GM6001 metalloproteinase inhibitor inhibits IL-10 production by CD46-activated T cells.** Purified $CD4^+$ T cells were left unstimulated, or stimulated by immobilized anti-CD3 or anti-CD3/CD46, as indicated, in presence of GM6001 or DMSO as control for 4 days. The proliferation was then assessed by thymidine incorporation, and the levels of IL-10 and IFNγ secreted in the culture supernatants were analyzed by ELISA.
(TIFF)

**Figure S2 Timely downregulation of expression of Cyt1 and Cyt2 upon T cell activation.** Purified $CD4^+$ T cells were left unstimulated, or stimulated by immobilized anti-CD3/CD46, as indicated, for 28–40 hrs (early time point) and 96–120 hrs (late time point). The expression of the two cytoplasmic tails of CD46 was determined by intracellular staining (0.1% saponin) using specific anti-Cyt1 or Cyt2 monoclonal antibodies (blue line), or isotype control (red line). The data obtained for three different donors are shown.
(TIFF)

**Figure S3 Inhibition of P/γS increases the levels of Cyt1/Cyt2 expression.** $CD4^+$ T cells were stimulated by immobilized anti-CD3/CD46 antibodies for 2 or 4 days in presence or absence of L-685,458, a P/γS inhibitor. The expression of Cyt1 and Cyt2 was then analyzed by flow cytometry. Addition of L-685,458 increases the levels of Cyt1 and Cyt2.
(TIFF)

**Figure S4 CTF expression can also alter the profile of cytokine produced and the proliferation of CD3-activated transfected T cells**. Twenty-four hours post transfection by

Amaxa/Lonza with the different CTF constructs, $CD4^+$ T cells were stimulated by immobilized anti-CD3 or anti-CD3/CD28 antibodies. (**A**) The secretions of IL-10 and IFNγ were assessed by secretion assays (Miltenyi). (**B**) Proliferation was assessed by flow cytometry (n = 8). In some experiments, anti-CD3 stimulation was very weak – hence we mainly studied the effects of expression of CTF in CD3/CD28 activated T cells. However, in the experiments where it induced T cell activation, we were then able to observe a similar effect of the CTF constructs.
(TIFF)

**Figure S5 Cyt2 inhibits proliferation of CTLA-$4^+$ cells.** Twenty-four hours post transfection by Amaxa/Lonza with the different CD19-CD46 fusion proteins, $CD4^+$ T cells were pre-labeled with CFSE and then stimulated by immobilized anti-CD3/CD28, anti-CD3/CD28/CD19 or anti-CD3/CD28/CD46 antibodies and CTLA-4 expression was determined after 4 days. The proliferation of CTLA-$4^+$ gated cells is shown.
(TIFF)

**Figure S6 Kinetics of Cyt1 and Cyt2 expression upon CD46-coactivation.** Purified $CD4^+$ T cells were left unstimulated (US), or stimulated by immobilized anti-CD3/CD46 (Stim), as indicated, for several days. The expression of the two cytoplasmic tails of CD46 was determined by intracellular staining using specific anti-Cyt1 or Cyt2 monoclonal antibodies (n = 5).
(TIFF)

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## Author Contributions

Conceived and designed the experiments: ALA SNC CN NJW MS. Performed the experiments: SNC CN JT NJW ALA. Analyzed the data: SNC CN ALA. Contributed reagents/materials/analysis tools: NJW MS JT. Wrote the paper: ALA.

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# Prostaglandin $E_2$ Affects T Cell Responses through Modulation of CD46 Expression

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# Prostaglandin $E_2$ Affects T Cell Responses through Modulation of CD46 Expression

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**The ubiquitous protein CD46, a regulator of complement activity, promotes T cell activation and differentiation toward a regulatory Tr1-like phenotype. The CD46-mediated differentiation pathway is defective in several chronic inflammatory diseases, underlying the importance of CD46 in controlling T cell function and the need to understand its regulatory mechanisms. Using an RNA interference-based screening approach in primary T cells, we have identified that two members of the G protein-coupled receptor kinases were involved in regulating CD46 expression at the surface of activated cells. We have investigated the role of $PGE_2$, which binds to the E-prostanoid family of G protein-coupled receptors through four subtypes of receptors called EP 1–4, in the regulation of CD46 expression and function. Conflicting roles of $PGE_2$ in T cell functions have been reported, and the reasons for these apparent discrepancies are not well understood. We show that addition of $PGE_2$ strongly downregulates CD46 expression in activated T cells. Moreover, $PGE_2$ differentially affects T cell activation, cytokine production, and phenotype depending on the activation signals received by the T cells. This was correlated with a distinct pattern of the $PGE_2$ receptors expressed, with EP4 being preferentially induced by CD46 activation. Indeed, addition of an EP4 antagonist could reverse the effects observed on cytokine production after CD46 costimulation. These data demonstrate a novel role of the $PGE_2$–EP4 axis in CD46 functions, which might at least partly explain the diverse roles of $PGE_2$ in T cell functions.** *The Journal of Immunology*, **2012, 188: 5303–5310.**

CD46 is a ubiquitously expressed type I membrane protein that was first identified as a regulator of the complement cascade, preventing autolysis of cells by binding to C3b/C4b and allowing their cleavage by protease I (1, 2). About 10 years ago, CD46 was shown to link innate immunity to acquired immunity. Indeed, costimulation of the TCR with CD46 leads to increased T cell proliferation (3) and affects T cell morphology (4) and polarity (5). Importantly, CD46 also drives Tr1 differentiation, characterized by secretion of high amounts of IL-10 (6) and granzyme B (7). IL-2 is key in CD46-mediated Tr1 differentiation, acting as a sensor to switch T cells from a Th1 to a Tr1 phenotype (8). The enzymatic processing of CD46 is a crucial feature of the CD46-mediated pathway that is involved in regulating T cell function. CD46 surface expression is strongly downregulated upon its own triggering, partly due to MMP cleavage of its ectodomain (9–11). This is followed by γ-secretase cleavage of the two cytoplasmic tails of CD46, which is important to initiate and terminate T cell responses (11, 12). This again underlines the importance of the plasticity of CD46 in controlling T cell homeostasis. Moreover, CD46-mediated Tr1 differentiation is altered in patients with multiple sclerosis (MS), characterized by an impaired IL-10 secretion upon CD3/CD46 costimulation (13–16), and the dysregulation of CD46 pathways in T cells was recently described in patients with asthma (17) and in a small group of patients with rheumatoid arthritis (RA) (8). The identification of a dysfunctional CD46 pathway in chronic inflammatory diseases highlights its importance in controlling T cell homeostasis and further underlines the need to understand its regulation and the molecular mechanisms responsible for its functions.

Using an RNA interference (RNAi)-based approach (18) to dissect the molecular pathways that regulate CD46 expression on primary human T cells, we identified two members of the serine/threonine kinase G protein-coupled receptor kinase (GRK) family involved in the regulation of CD46 expression. GRKs phosphorylate agonist-activated G protein-coupled receptors (GPCRs) (19, 20), resulting in their binding to β-arrestins and subsequent signaling impairment and internalization, a process known as desensitization (21, 22). There are seven types of GRK referred to as GRK 1–7, each with different expression profiles (21). Among them, GRK 2, 3, 5, and 6 are ubiquitously expressed, but are expressed at particularly high levels in immune cells, and have been shown to regulate inflammation (23).

In this study, we show that the knockdown of GRK2 and GRK3 strongly decreased CD46 expression and that activation of CD46 increased GRK2/3 expression levels. GRK2/3 have been shown to regulate $PGE_2$ receptors, among other GPCRs (24). As $PGE_2$ is a known modulator of T cell functions (25), we assessed the role of $PGE_2$ in the regulation of CD46 expression and function to demonstrate a role of GRKs in the CD46 pathway. $PGE_2$ notably inhibits T cell proliferation by downregulating both IL-2 and the IL-2Rα-

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; MFI, mean fluorescence intensity; MS, multiple sclerosis; RA, rheumatoid arthritis; RNAi, RNA interference; shRNA, short hairpin RNA; Treg, regulatory T cell.

chain (CD25) (26). $PGE_2$ can also markedly reduce production of Th1-associated cytokines such as IFN-γ, causing a switch from a Th1 to a Th2 cytokine secretion profile in these cells (26, 27). However, $PGE_2$ has also been shown to promote Th1 differentiation (28, 29) and to either decrease (30, 31) or promote IL-17 production (32–35). $PGE_2$ can also induce Foxp3 in naive $CD4^+$ T cells, with an increase in regulatory cell function (36). Hence, multiple effects of $PGE_2$ have been reported, and although the local concentrations of $PGE_2$ are important to control T cell differentiation (28), the reasons for these apparent discrepancies are not well understood. Moreover, there are no studies on any potential effects of $PGE_2$ on the CD46-mediated pathway. In this study, we first demonstrate that the addition of $PGE_2$ to T cell cultures strongly decreased CD46 expression in activated T cells. Second, we show that the addition of $PGE_2$ differentially affected T cell responses depending on the activation signals, as responses to CD28 and CD46 costimulation were different. Whereas T cell proliferation was decreased in all conditions, the IL-10/IFN-γ ratio was either increased or decreased depending on the activation status. Moreover, specific changes in the phenotype of activated T cells were observed. $PGE_2$ binds to the E-prostanoid family of GPCRs, through four subtypes of receptors called EP 1–4 (24). We show that CD46 and CD28 costimulation led to different patterns of expression of the two $PGE_2$ receptors, EP2 and EP4, with EP4 being preferentially expressed by CD46 activation. Overall, we demonstrate a novel role of the $PGE_2$–EP4 axis in CD46 functions and how this influences the effects of $PGE_2$ on human T cell activation.

## Materials and Methods

### *Abs and reagents used*

The Abs used for activation were anti-CD3 (OKT3; 5 μg/ml), anti-CD28 (CD28.2; 5 μg/ml), and anti-CD46 (20.6; 10 μg/ml), kindly provided by Dr. Chantal Rabourdin-Combe (INSERM, Lyon, France). $PGE_2$ (100 nM, unless otherwise stated) was from Sigma. Recombinant human IL-2 (Tecin) was added at 10 U/ml. The selective EP4 antagonist (ONO-AE2-227; 300 nM) was chemically synthesized by Charnwood Molecular (Leicester, U.K.). The EP2 antagonist (AH6809; 10 μM) was purchased from Calbiochem (Nottingham, U.K.). The Abs for flow cytometry were as follows: anti-CD46–FITC (clone MEM-258; BioLegend), anti-CD25–allophycocyanin (clone M-A251), anti-CD69–PE (clone FN50), and anti–CTLA-4–PE (clone BNI3) (BD Pharmingen). Anti-human Foxp3–allophycocyanin (clone PCH101; eBioscience) recognized all $Foxp3^+$ T cells (37). Anti–p-Tyr STAT3 (pY705)–Pacific blue was purchased from BD Biosciences. The Abs used for the GRK2/3 Western blot (rabbit) was purchased from Cell Signaling and used at 1 μg/ml (in 5% milk–TBST), followed by addition of anti-rabbit IgG–HRP from Promega (1:10,000).

### *Cell purification and activation*

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ) from heparinized venous blood from healthy donors obtained after informed consent. Ethical approval was obtained from the Lothian Board Ethics Committee. $CD4^+$ T cells were negatively isolated using magnetic beads (purification >90%; CD4 Isolation Kit II; Miltenyi Biotec, Auburn, CA). T cells ($1 \times 10^6$ cells/1 ml/well) were then cultured in 24-well plates precoated with anti-CD3, anti-CD28, or anti-CD46, in RPMI 1640 containing 10% FCS. Exogenous IL-2 (10 U/ml) was added to CD3/CD46 stimulated cells as previously described (6). In some experiments, $PGE_2$, selective EP2 or EP4 antagonists, and DMSO or ethanol as vehicle control (similar dilution) were added to the culture.

### *The RNAi Consortium RNAi library*

The complete description of the RNAi Consortium lentiviral RNAi library used in this study has been reported (38) and already used to infect primary T cells (18), and it is now commercialized by Sigma-Aldrich. Human genes are targeted with ~5 short hairpin RNAs (shRNAs) expressed under the control of the U6 Pol III promoter in a lentiviral vector (pLKO.1) that also confers puromycin resistance. Plasmid DNA purification and lentiviral production were performed as described [(38) and http://www.broad.mit.edu/rnai/trc/lib].

### *T cell stimulation and infection*

$CD4^+$ T cells ($2 \times 10^5$ cells/well) were cultured in 96-well plates precoated with anti-CD3 (OKT3; 2.5 μg/ml) and anti-CD28 (2D10; 2.5 μg/ml) in presence of human recombinant IL-2 (Tecin, 20 U/ml; National Cancer Institute, Frederick, MD) for 24 h before infection. The infection was carried out by centrifugation for 90 min at 2300 rpm at room temperature in presence of the viral supernatant and polybrene (8 μg/ml). After removal of the virus, fresh medium was added. Infected cells were then selected by addition of puromycin (2.5 μg/ml) 48 h postinfection, and the cells were expanded in IL-2 for 10 d. Cells were then restimulated with anti-CD3/CD28 Abs in the presence of IL-2. At 24 h post-restimulation, the level of CD46 expression at the cell surface was determined by flow cytometry.

### *GRK2/3 detection by Western blot*

Purified $CD4^+$ T cells were activated in 48-well plates precoated with anti-CD3, anti-CD46, or anti-CD28 Abs and cultured for 3 d. Cells were then lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, 10 mM NaF, 0.4 mM $Na_2VO_4$). Cell lysates were run on an 8% SDS-PAGE and amounts of GRK2 and GRK3 detected by Western blot using anti-GRK2 Abs that cross-react with GRK3 (Cell Signaling). The membranes were then stripped and reblotted with anti-GAPDH mAb to evaluate protein quantities. Densitometry analyses (with ImageJ) were then performed to calculate the GRK/GAPDH ratio.

### *Flow cytometry*

The expression level of CD46, CD25, and CD69 was assessed by flow cytometry with anti-CD46–FITC, anti-CD69–PE, and anti-CD25–allophycocyanin. The presence of CTLA-4 (PE) and Foxp3 (allophycocyanin) was determined after permeabilization of the cells with 0.1% saponin. The relative expression to the staining with the isotype control was calculated by calculating the Δmean fluorescence intensity (MFI) (MFI Ab stained − MFI control Ab). Samples were run on a BD FACSCalibur. Phosphorylation of STAT3 was analyzed with an anti–p-Tyr STAT3–Pacific blue and run on a BD FACSCalibur LSR Fortessa. All flow cytometry data were analyzed using FlowJo (Tree Star).

### *T cell proliferation*

Purified T cells ($2 \times 10^5$ cells/200 μl/well) were cultured in 96-well plates, precoated with anti-CD3, anti-CD28, or anti-CD46, in 10% FCS–RPMI 1640 for 72 h before addition of 1 μCi [$^3$H]thymidine (Amersham). Proliferation was determined using a Liquid Scintillation Counter (Wallac).

### *ELISA*

Cytokine production was determined in cell culture supernatants using ELISA specific for human IL-10 (BD Pharmingen, San Jose, CA) and IFN-γ (Endogen, Cambridge, MA). Recombinant hIL-10 (BD Pharmingen) and hIFN-γ (Endogen) were used as standards.

### *EP 1–4 PCR*

These PCRs were performed as previously described (39). Briefly, RNAs from activated T cells were extracted using the RNeasy Mini Kit (Qiagen). RNA samples were reverse-transcribed using VILO (Invitrogen, Paisley, U.K.) according to the manufacturer's guidelines. RT-PCR analysis of EP1, EP2, EP3, and EP4 was carried out using an ABI Prism 7500 (Applied Biosystems, Warrington, U.K.). Results are expressed relative to a standard (pooled normal human endometrial tissue cDNA) included in all reactions. Data are represented as mean ± SEM.

### *Statistical analyses*

The groups were analyzed using the GraphPad Prism software. Data were analyzed using the Wilcoxon test. All $p$ values are two-tailed and with a 95% confidence interval.

## Results

### *An RNAi screen identifies GRKs as novel regulators of CD46 expression in primary human T cells*

To gain new insights into the regulation of CD46 expression in primary human T cells, we carried out an RNAi screen using a subgenomic library targeting ~1000 genes, focused on kinases and phosphatases, and containing three to five shRNAs per gene, as previously described (18). Our goal was to identify novel

regulators of CD46 expression in primary human T cells. The level of CD46 expression at the cell surface of T cells infected with lentivirus particles expressing a distinct and unique shRNA was determined by flow cytometry. Using a statistical Z-score (see Ref. 18) to quantify the deviation of CD46 levels from the mean of all measurements within the same plate, we selected shRNAs that significantly modulated CD46 expression compared with the cohort of control wells consisting of cells infected with control shRNAs (against nonmammalian reporter genes) (Fig. 1A). GRK2 and GRK3, two members of the GRK family, decreased CD46 expression once knocked down (Fig. 1A, circled). Two shRNA constructs out of four for GRK2 and two out of three for GRK3 led to a similar phenotype (Fig. 1A). These data were reproduced in two independent experiments performed with different donors (Fig. 1B). Representative plots showing normal CD46 expression compared with expression upon GRK2 and GRK3 knockdowns are shown in Fig. 1C. Hence, downregulation of GRKs decreased CD46 expression in activated primary human T cells.

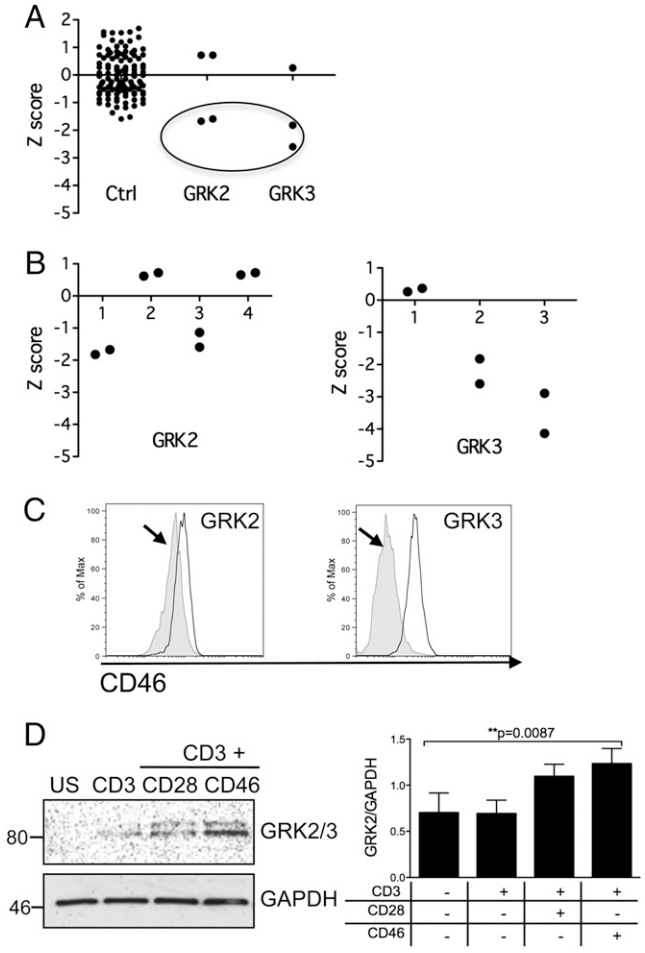


**FIGURE 1.** GRKs regulate CD46 expression in primary T cells. (**A**) An RNAi screen was performed on primary $CD4^+$ T cells infected by lentivirus coding for 5000 different shRNA (1 shRNA construct per well). The Z-score obtained after knockdown of GRK2 and GRK3 compared with controls is shown. Infection by two different shRNA constructs targeting GRK2 and GRK3 led to strong negative Z-scores, corresponding to a decrease in CD46 expression (circled). (**B**) The Z-scores obtained across two independent experiments with different donors for the multiple shRNAs targeting GRK2 and GRK3 are shown. The phenotypes observed were repeatedly due to the same hairpins. (**C**) CD46 expression measured by flow cytometry on cells infected with control lentiviruses or shRNA for GRK2 and GRK3. Arrows point to the constructs inducing CD46 downregulation. (**D**) CD46 activation induces GRK2/3. $CD4^+$ T cells were left unstimulated (US) or were activated with immobilized anti-CD3, anti-CD3/CD28, or anti-CD3/CD46 Abs for 3 d. Cell lysates were obtained and GRK2/3 levels determined by Western blot. Quantification was obtained after stripping of the membrane and reblotting with GAPDH Abs. The GRK/GAPDH ratio obtained for four donors is also represented.

### *CD46 activation led to enhanced GRK2/3 levels*

T cell activation by PHA and anti-CD3 Abs increases GRK expression and activity (40, 41). Hence, we next assessed whether CD46 costimulation could modulate GRK2/3 levels. $CD4^+$ T cells were activated by immobilized anti-CD3, anti-CD3/CD28, or anti-CD3/CD46 Abs for 3 d. GRK2/3 levels were then assessed by Western blots using anti-GRK2/3 Abs. T cell activation by CD28 and especially CD46 led to enhanced levels of GRKs (Fig. 1D). Hence, these data demonstrate a relationship between GRKs and the CD46 pathway.

### *$PGE_2$ decreases CD46 surface expression in activated T cells*

$PGE_2$ is a known T cell modulator that signals through binding to the E-prostanoid family of GPCRs (namely EP 1–4). GRK2 regulates expression of the EP4 receptor (24), and we found that EP4 knockdown led to increased CD46 expression (Supplemental Fig. 1), suggesting a role of $PGE_2$ in controlling CD46 expression. Hence, to assess whether signaling through GPCR/GRK could modulate CD46 expression, we next investigated the effect of $PGE_2$ on CD46 expression and function. $CD4^+$ T cells were activated with anti-CD3, anti-CD3/CD46, or anti-CD3/CD28 Abs in the presence of $PGE_2$ or DMSO as a vehicle control, and expression of CD46 was monitored by flow cytometry. Fig. 2A shows the representative data obtained after 2 d of culture for one donor, and Fig. 2B shows the average data obtained with the different donors after 2 or 5 d of culture ($n = 15$). The percentage changes in CD46 expression upon addition of $PGE_2$ (compared with expression levels in absence of $PGE_2$ as baseline) were also calculated and are presented in Fig. 2C. As previously shown, CD46 ligation led to a strong downregulation of its expression. A slight increase in CD46 surface expression was detected when CD46 and CD28 were co-ligated, suggesting that there is a cross-talk between CD28 and CD46 that partially restores CD46 levels (Fig. 2B). The addition of $PGE_2$ led to a slight increase in CD46 expression in unstimulated T cells after 2 d of culture (Fig. 2B, 2C). In contrast, $PGE_2$ treatment led to decreased CD46 expression in activated T cells with the stronger reduction (~40%) observed upon CD46 costimulation. The effect of $PGE_2$ was lost in CD3-activated T cells at day 5 (Fig. 2B). However, the decrease in CD46 expression was sustained by CD28 and CD46 costimulation, with again the stronger effect observed upon CD46 ligation. Fig. 2D shows the dose-dependent effect of $PGE_2$ on CD46 expression. Hence, $PGE_2$ clearly modulates CD46 expression in activated T cells.

### *$PGE_2$ differentially affects T cell activation depending on the costimulation signal*

We next assessed the role of $PGE_2$ on T cell activation. $CD4^+$ T cells were activated with anti-CD3, anti-CD3/CD46, or anti-CD3/CD28 Abs in the presence or absence of $PGE_2$. First, T cell proliferation was determined by [$^3$H]thymidine incorporation ($n = 10$). As expected, addition of $PGE_2$ significantly decreased T cell proliferation in a dose-dependent manner, but the strongest effect was observed upon CD46 costimulation (Fig. 3A, 3B). Similar effect was observed when proliferation was determined by CFSE labeling (data not shown). Second, we determined whether the levels of cytokine produced by activated T cells were modified by addition of $PGE_2$. We focused on IL-10 and IFN-γ as CD46 has the ability to switch T cells from producing IFN-γ to

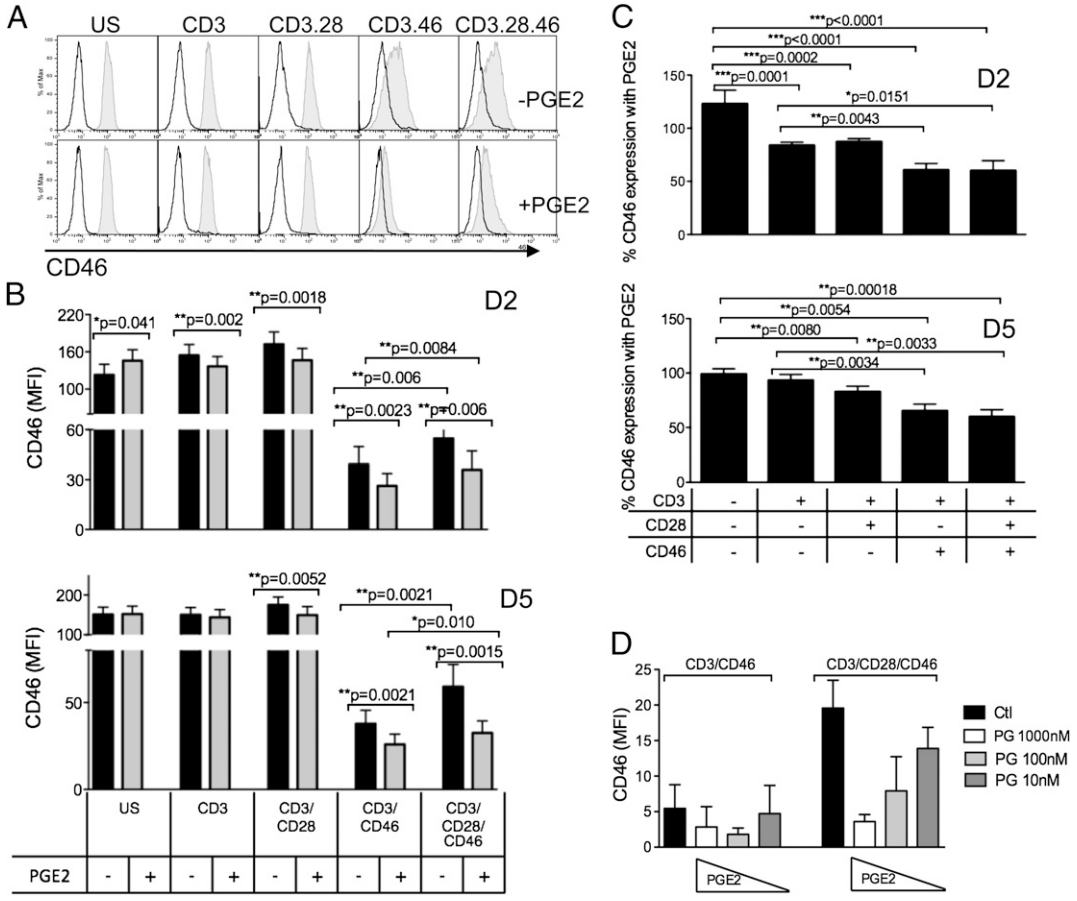


**FIGURE 2.** $PGE_2$ regulates CD46 expression. (**A**) Purified $CD4^+$ T cells were left unstimulated (US) or stimulated as indicated by immobilized Abs in the presence of $PGE_2$ or DMSO as vehicle control. CD46 expression was then monitored by flow cytometry after 2 d (CD46 staining: shaded gray areas; isotype control: black lines). (**B**) The expression of CD46 was monitored in T cells cultured for either 2 or 5 d in the presence or absence of $PGE_2$ ($n = 15$). (**C**) The percentage changes in CD46 expression upon addition of $PGE_2$ compared with expression levels in the absence of $PGE_2$ as baseline are represented. Samples were analyzed using the Wilcoxon test. The means ± SEM are shown ($n = 15$ donors). (**D**) The expression of CD46 was monitored in T cells cultured for 3 d in the presence of increasing doses of $PGE_2$, as indicated ($n = 3$).

secreting IL-10 (8) and does not induce any Th2 cytokines (6, 42). Secretion of IL-10 and IFN-γ was quantified by ELISA, and we also calculated the IL-10/IFN-γ ratio to bypass the variations due to the changes in proliferation ($n = 15$). As previously described, CD46 activation promotes a Tr1-like phenotype, visualized by an increase in the IL-10/IFN-γ ratio (Fig. 3C). $PGE_2$ drastically decreased cytokine production (Fig. 3C). However, $PGE_2$ had different effects on the relative levels of cytokines produced depending on the activation signals received by the T cells. A trend toward an increase in IL-10/IFN-γ was observed upon CD3 activation alone, reflecting the known effect of $PGE_2$ in the downregulation of IFN-γ. Costimulation with CD28 had no significant effect on the IL-10/IFN-γ ratio, suggesting that the lower level of cytokine production was mainly correlated with the lower proliferation of the cells. In contrast, ligation of CD46 resulted in a significant decrease in the IL-10/IFN-γ ratio in the presence of $PGE_2$. Moreover, the effects of CD46 were dominant over CD28 as also observed upon CD3/CD28/CD46 co-ligation. Importantly, similar trends were obtained when naive $CD4^+$ T cells were used (data not shown). Overall, these data indicate that $PGE_2$ exerts different effects on cytokine production that mainly depend on the signals received by T cells.

*$PGE_2$ modulates the phenotype of CD46-costimulated T cells*

We next determined whether $PGE_2$ was affecting the phenotype of CD46-costimulated T cells. As $PGE_2$ is known to reduce CD25 expression in CD3-activated T cells (36), we assessed the expression levels of CD25 as a control. We also determined the levels of CD69, another activation marker (Fig. 4A). Moreover, as $PGE_2$ has been reported to induce regulatory T cells (Tregs) (36), we determined the expression levels of CTLA-4 (Fig. 4A), Foxp3, and CD46 (Fig. 4B). Fig. 4C represents the average change in expression upon addition of $PGE_2$ obtained for the different donors ($n = 6$). A strong decrease in CD25 expression was detected for all conditions of activation, including CD46-costimulated T cells. Surprisingly, whereas CD69 expression was strongly decreased in the presence of $PGE_2$ upon CD3 and CD28 activation, its expression was maintained when the cells were costimulated by CD46. T cell activation in the presence of $PGE_2$ also resulted in a decrease in CTLA-4 expression, which was slightly restored upon costimulation. Lastly, $PGE_2$ increased Foxp3 expression in both CD28 and CD46 costimulated T cells. Notably, the cells acquiring Foxp3 maintained CD46 expression although CD46 expression in CD46-costimulated T cells was downregulated by $PGE_2$ (Fig. 4B). These data show that, depending on the activation signals, $PGE_2$ differentially affects a variety of phenotypic markers in T cells that are involved in regulating T cell activation as well as Treg functions.

*$PGE_2$ inhibits STAT3 phosphorylation*

$PGE_2$ decreased CD46 expression in activated T cells, and it has been shown to modulate STAT3 signaling (43, 44). As the CD46

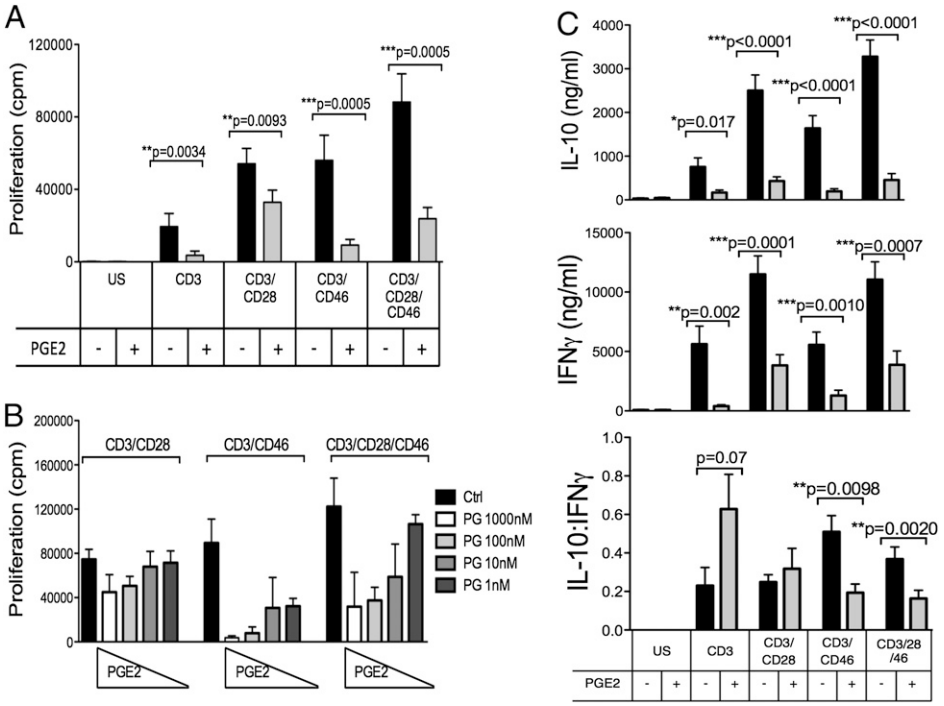


**FIGURE 3.** $PGE_2$ modulates T cell activation depending on the activation signals. Purified $CD4^+$ T cells were stimulated by immobilized Abs as indicated in the presence or absence of $PGE_2$. (**A**) Proliferation was determined after 4 d by [$^3$H]thymidine incorporation ($n$ = 10). (**B**) The dose effect of $PGE_2$ on T cell proliferation of T cells activated by anti-CD3/CD28, anti-CD3/CD46, or anti-CD3/CD28/CD46 is shown ($n$ = 3). (**C**) Cells were activated as in (A), and the amounts of IL-10 and IFN-γ in the supernatants were determined by ELISA ($n$ = 15). The IL-10/IFN-γ ratio is also represented to show specific effects independent of the changes in proliferation induced by $PGE_2$.

promoter includes a STAT3 binding site, and as the direct interaction between STAT3 and CD46 has been previously demonstrated by chromatin immunoprecipitation (ChIP) assays, mutations of the STAT3 binding sites in CD46, and by the use of anti-STAT3 oligonucleotides (45, 46), we determined the levels of STAT3 phosphorylation in activated T cells. T cells were activated for 24 h in the presence or absence of $PGE_2$ and STAT3 phosphorylation determined by flow cytometry. An increase in phosphorylation was observed in all activated T cells, including CD46-costimulated T cells. Addition of $PGE_2$ decreased STAT3 phosphorylation, with the strongest effect observed upon CD46 ligation (Fig. 5).

### *Specific changes in $PGE_2$ receptors are induced by CD46 activation*

$PGE_2$ binds to four GPCRs, namely EP1, EP2, EP3, and EP4. We determined their mRNA expression by quantitative PCR upon

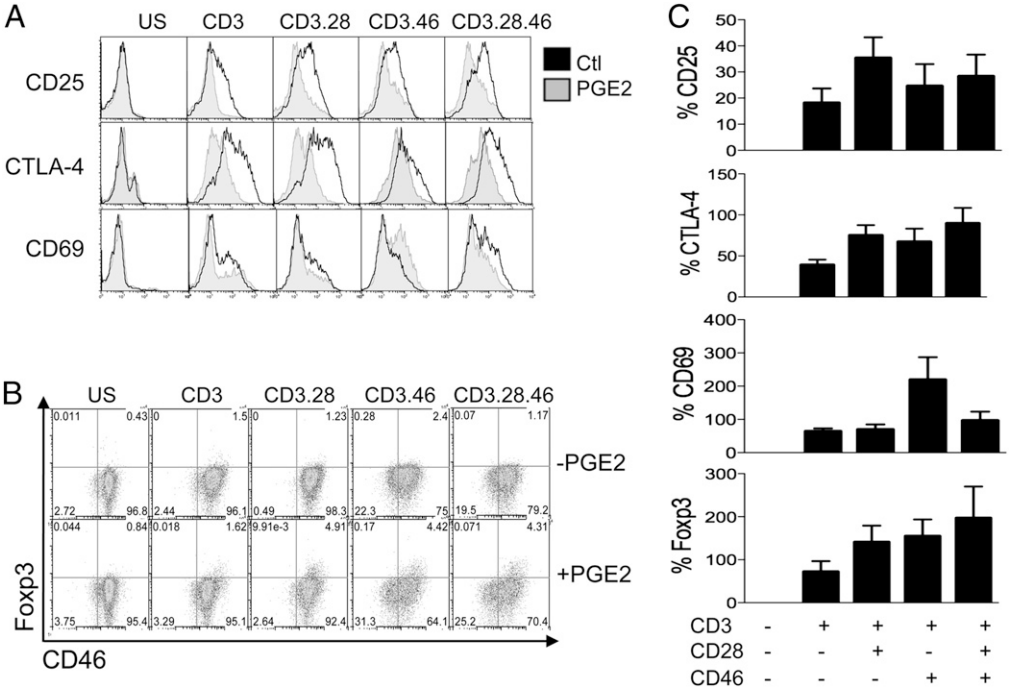


**FIGURE 4.** $PGE_2$ modulates T cell phenotype upon activation. (**A**) Purified $CD4^+$ T cells were stimulated with immobilized anti-CD3, anti-CD3/anti-CD28 (CD3.28), anti-CD3/CD46 (CD3.46), or anti-CD3/CD28/CD46 (CD3.28.46) Abs in the presence (shaded gray histograms) or absence (black line) of $PGE_2$. After 5 d, the expression of surface CD25 and CD69 and intracellular CTLA-4 was determined by flow cytometry. (**B**) Expression of Foxp3 and CD46 was also determined after intracellular staining. (**C**) The percentage changes in CD25, CTLA-4, CD69, and Foxp3 expression upon addition of $PGE_2$ obtained for the different donors are represented ($n$ = 6).

T cell activation with anti-CD3, anti-CD3/CD28, and anti-CD3/CD46 Abs for 48 h ($n$ = 3 different donors, and experiments performed at different times). As previously reported (33), only EP2 and EP4 were strongly expressed in T cells. Whereas CD3 and CD28 costimulation induced both EP2 and EP4 expression (Fig. 6A), CD46 activation only led to an increased EP4 expression, hence strongly increasing the EP4/EP2 ratio (Fig. 6B).

To understand the role of EP4 in CD46 activation, we added the selective EP4 antagonist ONO-AE2-227 to T cell culture and assessed cytokine production. Blocking EP4 led to an increase in the IL-10/IFN-γ ratio, but only when CD46 was ligated, and we did not observe any effect of the EP2 antagonist AH6809 (Fig. 6C). This further demonstrates the role of the PGE$_2$–EP4 axis in regulating IL-10 production by CD46-activated T cells.

## Discussion

This study reports novel elements in the complex regulation of primary human T cell activation. Our data show that GRKs and the PGE$_2$–EP4 axis are involved in the CD46 pathway and that it modulates CD46 expression and functions. Knocking down GRKs led to a strong downregulation of CD46 at the surface of activated T cells. It is known that GRK levels are modulated by T cell activation (40, 41), and we report in this study that CD46 costimulation increased levels of GRK expression. Notably, decreased levels of GRK2 and GRK6 were observed in T cells from patients with RA (47) and MS (48, 49), and proinflammatory cytokines and oxygen radicals can decrease GRK2 levels in vitro (50). Hence, the levels of GRKs are highly modulated during inflammation, and this suggests the subsequent modulation of expression of CD46, a key regulator of T cell activation, in inflammatory settings. As CD46 is dysfunctional in T cells from patients with MS and RA, future investigation into the role of GRKs upon CD46 activation in these patients might highlight some of the molecular mechanisms of this defective pathway.

GRKs control GPCR signaling by phosphorylating these receptors, which provokes their internalization and degradation, a crucial feature of the plasticity of the immune system involved in the regulation of inflammation (reviewed in Ref. 23). Indeed, almost 30% of drugs on the market target GPCR (51, 52). We investigated the role of PGE$_2$, which signals through four subtypes of the E-prostanoid subfamily of GPCRs (EP1, EP2, EP3, and EP1–4) in T cell responses. Although the antiproliferative role of PGE$_2$ on T cells is well established, the role of PGE$_2$ in T cell differentiation is much more complex and not well understood, as it has some complex proinflammatory and immunoregulatory properties. In this study, we have compared the effects of PGE$_2$ in CD28 and CD46 costimulated T cells. As previously shown, PGE$_2$ exerted a strong antiproliferative role on all activated T cells. However, the effect was much stronger in CD46-costimulated T cells than in CD28-coactivated T cells. Despite the decrease in proliferation, activated T cells did secrete some significant

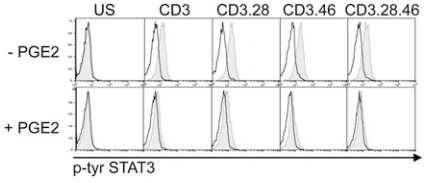


**FIGURE 5.** PGE$_2$ inhibits STAT3 phosphorylation. Purified CD4$^+$ T cells were stimulated by immobilized Abs as indicated in the presence or absence of PGE$_2$. After 24 h, the level of p-Tyr STAT3 was determined by flow cytometry (representative of two independent experiments). (STAT3 staining: shaded gray areas; isotype control: black lines.)

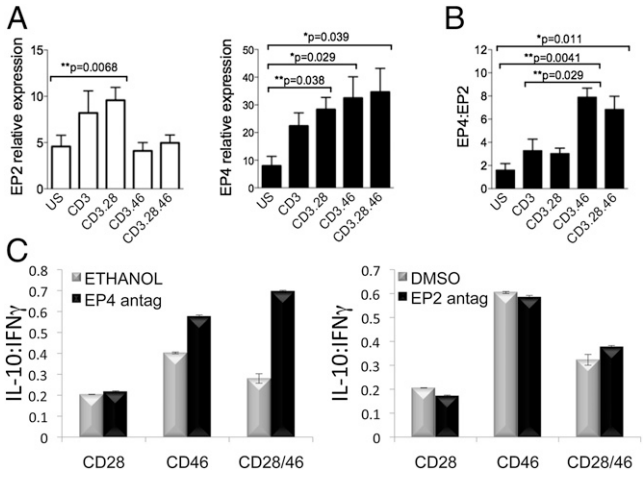


**FIGURE 6.** T cell activation results in expression of different PGE$_2$ receptors depending on the activation signal. Purified CD4$^+$ T cells were stimulated by immobilized Abs as indicated for 2 d. mRNA was then prepared and the expression levels of the four PGE$_2$ receptors (EP1–4) determined. (**A**) Relative expression of EP2 and EP4 in activated T cells. The means ± SEM are shown ($n$ = 3 donors). Samples were analyzed using the Wilcoxon test. (**B**) The ratio of EP4/EP2 relative expression has been plotted. CD46 activation results in an increased EP4/EP2 ratio. (**C**) EP4 is involved in the modulation of cytokines produced by CD46-costimulated T cells. Purified CD4$^+$ T cells were stimulated by immobilized Abs as indicated in the presence of EP2 or EP4 antagonist and DMSO or ethanol as a control, respectively. The levels of IL-10 and IFN-γ were quantified, and the IL-10/IFN-γ ratio is represented. Representative of three experiments.

levels of IL-10 and IFN-γ. In a number of studies, the addition of PGE$_2$ caused a marked reduction in Th1 cytokines (26, 27), whereas other studies indicate the role of PGE$_2$ in inducing Th1 or Th17 differentiation (28, 33). We found that the relative levels of IL-10 and IFN-γ secreted were differentially modulated depending on the activation signals received by the T cells. Whereas CD3 activation favored an increase in the IL-10/IFN-γ ratio, as previously described, we did not find any significant effects on CD28-costimulated T cells. However, there was a significant decrease in the IL-10/IFN-γ ratio upon CD46 ligation. PGE$_2$ therefore skews the usually regulatory cells toward a more inflammatory secretion profile. PGE$_2$ downregulates both IL-2 and CD25 (26). As IL-2 is required for CD46-mediated Tr1 differentiation (8), it is likely that the effect of PGE$_2$ on IL-2 and CD25 expression as well as the inhibition of CD46 expression contribute to inhibit IL-10 production by CD46-activated T cells. Our data also underline that the actions of PGE$_2$ are largely dependent on the activation of the T cells, as previously reported on the secretion of IL-3 by T cells (53). Hence, it becomes clear that PGE$_2$ differentially modulates T cell responses depending on the environment and signals received by the T cells. Our data highlight the role of PGE2 in the production of IFN-γ and IL-10, which are, at least in part, determined by the regulation of expression of CD46.

PGE$_2$ strongly modulates the phenotype of activated T cells. We show that the addition of PGE$_2$ decreased CD46 expression. This was observed in all activated T cells, although the effect was much more pronounced upon CD46 ligation. Of note, we observed a slight increase in CD46 expression in unactivated T cells. Notably, PGE$_2$ has been found to upregulate CD55, another regulator of complement activation molecule, in colon cancer cells (54). Hence, there is a similar pattern for CD46 in resting T cells. However, the increased expression of CD55 might also partially counteract the downregulation of CD46 expression observed in

activated cells to protect from complement attack. Importantly, as CD46 is ubiquitously expressed, it is likely that $PGE_2$ will also modulate its expression and function in other cell types, such as dendritic cells, especially as $PGE_2$, like CD46, has been shown to regulate IL-23 production (35, 55).

It was previously demonstrated that $PGE_2$ decreased CD25 expression and upregulated Foxp3 levels in human T cells (36). We confirmed these findings and report that similar decreased CD25 and increased Foxp3 expression are also detected after CD46 costimulation. Moreover, $PGE_2$ caused a strong reduction of expression of the coinhibitory molecule CTLA-4 on CD3-activated cells, but CTLA-4 expression was mostly maintained upon costimulation. CTLA-4 is expressed at high levels by Tregs and is involved in their function (56), hence the increase in Foxp3 correlates with CTLA-4 expression. $PGE_2$ also decreased CD69 expression in activated T cells, although not in CD46-costimulated cells. CD69 is transiently induced after activation but can persist on leukocyte infiltrates in chronic inflammatory diseases. CD69-deficient mice developed exacerbated forms of arthritis, allergic asthma, and other inflammatory settings, and it was proposed that CD69 could act as a regulator of Th17 differentiation (57). The fact that $PGE_2$ maintained CD69 in CD46-costimulated T cells is intriguing and warrants further investigation on this pathway, notably in chronic inflammatory diseases where the CD46 pathway is dysfunctional.

The different effects on cytokine production and phenotype observed in the presence of $PGE_2$ were correlated with distinct profiles of expression of $PGE_2$ receptors. CD3 and CD28 led to similar increase in EP2 and EP4, which is consistent with what had been shown in the mouse (58) and in human T cells (33). However, CD46 costimulation largely favored EP4 expression, and addition of an EP4 antagonist increased the IL-10/IFN-γ ratio by CD46-activated T cells, highlighting the specific role of EP4 in CD46 function. Contrasting effects of $PGE_2$ on cytokine production have previously been shown to be mediated by specific receptors. Boniface et al. (33) show that $PGE_2$ can act on CD3/CD28/CD2-coactivated human T cells to promote IL-17, which is mainly mediated by EP2, whereas it inhibits IFN-γ and IL-10 production mainly through EP4 signaling. Both the EP2 and the EP4 receptors couple with the Galpha(s) protein, whereas the EP1 and the EP3 receptors are known to couple to Galpha(q) and Galpha(i), respectively. However, differences in signaling between EP2 and EP4 have indeed been demonstrated, as for example EP4 but not EP2 leads to ERK phosphorylation via PI3K activation (59). It is likely that different signaling cascades are involved in the CD28 and CD46 pathways. The link between CD46 and EP4 is also intriguing as there is a special emphasis on these receptors in MS. Among $PGE_2$ receptor-deficient mice, only the EP4-subtype knockout can significantly inhibit experimental autoimmune encephalomyelitis (29). Moreover, MS-associated single nucleotide polymorphisms in EP4 (also called PTEGR4) have been demonstrated (60). As it is now clearly established that the CD46 pathway is dysfunctional in MS (13–16), further investigations should address the question as to whether the CD46–$PGE_2$–EP4–GRK pathway is dysfunctional in MS.

Hence, we propose a model in which $PGE_2$, secreted at sites of inflammation and possibly by T cells themselves (61, 62), binds to EP4, the specific $PGE_2$ receptor induced by CD46 activation. This initiates a signaling cascade that results in decreasing CD46 expression, which might be, at least in part, through STAT3 inhibition. Further studies using STAT3 ChIP experiments could confirm the role of $PGE_2$ in the regulation of CD46 transcription by STAT3. GRKs are also induced by CD46 costimulation and will then bind to EP4 and terminate response through internalization of the $PGE_2$ receptor. Of note, whereas EP4 couples via arrestin upon ligand binding, EP2 does not and is resistant to agonist-induced desensitization (63). Therefore, it seems logical that CD46 would favor EP4 that can be desensitized to ensure that proper levels of CD46 expression are restored. When GRKs are knocked down, the $PGE_2$–EP4 signaling goes on resulting in a continuous decrease in CD46 expression.

The effects of $PGE_2$ are known to be complex, depending on the site and timing of release, and the cell on which it acts. Our data demonstrate the novel role of the $PGE_2$–EP4–GRK axis in CD46 functions and how this influences the effects of $PGE_2$ on cytokine production and expression of several markers involved in T cell regulation. The many complex and interlinked actions of $PGE_2$ on lymphocytes and on other immune cells will require further investigation to decipher fully the complexity of this system.

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## Disclosures

The authors have no financial conflicts of interest.

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